short sleep duration and the development of proteinuria, but this association was not assessed in the present study.

In conclusion, the present study identified short sleep duration (≤5 hours) as a significant predictor of proteinuria, even adjusting for multiple clinically relevant metabolic and lifestyle factors. These results provide novel insight into the mechanism of the development of proteinuria, which is one of the critical predictors of end-stage renal disease and CVD.

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REVIEW ARTICLE

The 36th IUPS Satellite Symposium:
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Towards developing new strategies to reduce the adverse side-effects of nonsteroidal anti-inflammatory drugs

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Abstract The antipyretic and analgesic actions of nonsteroidal anti-inflammatory drugs (NSAIDs) are caused by the inhibition of prostaglandin E_2 (PGE_2), thromboxane A_2 and prostacyclin (PGI_2) production. Accumulating evidence suggests that the inhibition of PGE_2 production can cause adverse side-effects of NSAIDs on fluid and blood pressure regulation, such as hypertension and edema formation. Since both cyclooxygenase (COX)-1 and COX-2 isoforms contribute to the production of PGE_2, selective COX-2 inhibitors are not free of these adverse side-effects although they may be less severe. Four subtypes of PGE_2 receptors have been identified. The antipyretic action of blunted PGE_2 production is mediated predominantly by a reduced input to the prostaglandin E receptor 3 (EP_3) pathway, whereas the analgesic action is mediated predominantly by a reduced input to the EP_1 pathway and perhaps by contributions from the other EP receptors. Accordingly, some of the adverse side-effects might be moderated by combined use of NSAIDs with selective EP_2 or EP_4 agonists that do not block the antipyretic or analgesic actions of NSAIDs that are mediated by reduced activation of EP_1 or EP_3 receptors. Moreover, EP_2 receptor-deficient mice had salt-sensitive hypertension and EP_4 receptor blockade moderated salt and water excretion and both EP_2 and EP_4 agonists had renoprotective effects. This suggests that strategies to maintain activation of EP_2 and EP_4 receptors during NSAID administration may not only reduce adverse effects but might confer additional benefits. In conclusion, enhancing EP_2 and EP_4 receptor activity by administration of selective agonists during the administration of NSAIDs has the potential to permit treating fever, inflammation and pain but with marginal adverse effects on fluid or blood pressure regulation.

Keywords COX-1 · COX-2 · PGE_2 · EP · Antipyretic · Analgesic

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for their antipyretic and analgesic actions. The pharmacological action of NSAIDs originates from the inhibition of cyclooxygenase (COX). COX converts arachidonic acid to prostaglandin H_2, which is further metabolized enzymatically into five different prostanoids: thromboxane A_2 (TxA_2), prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin F_2 (PGF_2), and prostaglandin D_2 (PGD_2) [1, 2]. These prostaglandins mediate diverse biological actions some of which are antagonistic to each other. For example, TxA_2 promotes vasoconstriction, whereas PGI_2 has vasodilatation activity [3]. Moreover, COX has two isoforms, named COX-1 and COX-2. Although the classical NSAIDs inhibit both COX-1 and -2

activities non-selectively, the affinities of NSAIDs for each COX isoform are often different [4]. This makes it difficult to predict the clinical impact of NSAIDs. The more recently developed selective COX-2 inhibitors will be discussed later in this review.

Pharmacological mechanisms underlying the antipyretic and analgesic action of NSAIDs

The mechanism of fever has been investigated utilizing lipopolysaccharide (LPS)-infused animal models. Inhibition of PGE_2 production was established as the major mediator of the antipyretic action of NSAIDs [5]. An increase of PGE_2 in cerebrospinal fluid followed by activation of the EP_3 (and possibly EP_1) receptor in the ventromedial preoptic-anterior hypothalamus (POA, the locus of the temperature regulating center) is firmly linked to the development of fever [6–9]. It remains uncertain whether PGE_2 produced in peripheral tissues, especially in liver Kupffer cells, can directly stimulate fever. Moreover, Gao et al. [10] have shown that peripheral PGD_2 production is also significant for the development of fever. Thus, the pyretic action of prostaglandins is mediated predominantly by EP_3 receptors, but there can be contributions mediated by EP_1 receptors and PGD_2.

In contrast, the analgesic actions of NSAIDs are mediated by multiple receptors, including EP_1, EP_2, EP_3, IP, and likely EP_4. Moriyama et al. [11] have shown that PGE_2 and PGI_2 induced pain by activating the capsaicin-sensitive transient receptor potential vanilloid I (TRPVI) in sensory nerves through actions on EP_1 and prostaglandin I (IP) receptors. This is mediated by the activation of a protein kinase C-dependent pathway. This group has shown further that EP_4 receptors can potentiate TRPVI receptor activation via a protein kinase A (PKA)-dependent pathway. Reinold et al. [12] reported the EP_2 receptor to mediate spinal and peripheral inflammatory hyperalgesia. Finally, Ueno et al. [13] demonstrated the importance of EP_3 and IP receptors in the acetic acid-induced writhing model of pain in animals pretreated with LPS. Thus, a wide range of prostaglandin receptors have been implicated in mediating pain responses in different animal models. Further work is required to establish the relative importance and interactions between these receptors.

Pharmacological mechanisms of NSAIDs on fluid and blood pressure status

Gastrointestinal mucosal damage [14], cardiovascular events due to venous thrombosis and pulmonary embolism [4], renal dysfunction [15], hypertension, and edema formation have been recognized as adverse side-effects of NSAIDs. Among these complications, this article will focus on the effects of NSAIDs on fluid and blood pressure (BP) regulation. It has been reported that NSAIDs can affect fluid and BP status by modulating vascular tone of resistant vessels, renin release, vasopressin signaling, and renal tubular sodium handling.

1. *Vascular tone of resistant vessels*. TxA_2 produced in endothelial cells activates thromboxane prostanoid receptors on vascular smooth muscle cells and promotes vasoconstriction [16], whereas PGI_2 produced in endothelial cells antagonizes TxA_2 action [17]. The activation of EP_1 and EP_3 receptors results predominately in vasoconstriction, whereas the activation of EP_2 and EP_4 receptors results in vasodilatation through the activation of a PKA-dependent pathway [18–20].
2. *Renin release*. COX-2 action in macula densa cells enhanced renin release from juxtaglomerular (JG) granular cells [21]. PGE_2 and PGI_2 production, catalyzed by COX-2, activated IP, EP_2, and EP_4 receptors in JG cells and promoted the exocytosis of renin granules through the activation of the PKA-dependent pathway [22].
3. *Vasopressin signaling*. Prostaglandins can counter the actions of vasopressin. Therefore, NSAIDs can enhance vasopressin effects in the kidneys. PGE_2 is reported to be a key inhibitor of the antidiuretic action of vasopressin mediated via the vasopressin-2 (V_2) receptor. Vasopressin increased the phosphorylation of the aquaporin-2 (AQP2) protein at the cytoplasmic COOH terminus at serine 256 through the activation of the V_2 receptor/PKA-dependent pathway [23]. Phosphorylated AQP2 is the active form of the water channel which is recruited to the apical membrane to increase water reabsorption from the tubule lumen into the bloodstream. This leads to urinary concentration. Melvin et al. [24] proposed that the EP_3 receptor inhibited vasopressin signaling by counteracting PKA activation. Li et al. [25] reported that the activation of the EP_4 receptor in V_2 receptor-deficient mice moderated the polyuria by increasing the expression of the renal AQP2 protein via an activated cAMP/PKA-dependent pathway. These results demonstrate that the EP_3 receptor is required for full inhibition of vasopressin V_2 receptor signaling and that the EP_4 receptor can further enhance free-water reabsorption independent of vasopressin and V_2 receptor expression.
4. *Renal tubular sodium handling*. A significant role for PGE_2 in renal tubular sodium handling has been demonstrated in studies of children with hyperprostaglandin E syndrome/antenatal Bartter syndrome (HPS/aBS) [26]. Affected infants presented with polyuria and salt wasting attributed to impaired tubular

reabsorption in the thick ascending limb. PGE_2 formation was increased in these patients and COX inhibitors, such as indomethacin, reduced polyuria and salt wasting. Chen et al. [27] demonstrated that renal medullary EP_2 receptors participated in natriuresis in mice during a high-salt diet. Nüsing et al. [28] generated a mouse model of HPS/aBS using prolonged administration of furosemide. They reported the involvement of EP_1, EP_3, and especially EP_4 receptor activation in the enhanced natriuresis in this model. Therefore, all four EP receptors have been implicated in promoting natriuresis in particular situations.

Can selective COX-2 inhibitors moderate the adverse side-effects of NSAIDs on fluid and BP regulation without affecting antipyretic and analgesic action?

COX-2 was isolated as a form of COX that was induced by inflammation [29]. Accordingly, selective COX-2 inhibitors were anticipated to retain anti-inflammatory actions but to have a lesser risk of adverse side-effects than classical NSAIDs. Selective COX-2 inhibitors were reported in earlier studies to have a somewhat reduced risk of causing gastrointestinal bleeding, but the risk of developing cardiovascular events was similar to classical NSAIDs [4]. Moreover, selective COX-2 inhibitors promoted edema formation and BP elevation [30, 31]. Thus, the proposed advantages of selective COX-2 inhibitors were not confirmed. One of the reasons may be that COX-2 is constitutively expressed and participates in normal physiological actions. For example, COX-2 is expressed constitutively in the renal medulla and renal macula densa cells where it affects renin secretion [32, 33]. Moreover, COX inhibitors exert their antipyretic and analgesic actions by inhibiting PGE_2 production, yet reduced PGE_2 is now recognized as a major cause of the adverse effects of NSAIDS on fluid and BP regulation. Each COX isoform is coexpressed with specific prostaglandin synthases. This may explain why COX-1 produces predominantly TxA_2 and PGE_2 [34–36], whereas COX-2 produces predominantly PGI_2 and PGE_2 [37, 38]. Thus, both COX isoforms contribute to the production of PGE_2.

How can the adverse side-effects of NSAIDs on fluid and BP regulation be moderated without affecting antipyretic and analgesic action?

Among the four PGE_2 receptor subtypes, the EP_1 and EP_3 receptors contribute predominantly to the antipyretic and analgesic actions. This raises the possibility that some of the adverse side-effects of NSAIDs might be moderated by coadministration of selective EP_2 or EP_4 agonists that would not block the antipyretic or analgesic actions. Vukicevic et al. [40] reported renoprotective effects of both EP_2 and EP_4 agonists in an acute kidney injury model produced by mercury chloride injection. While it is well established that EP_2 or EP_4 receptors promote vasodilatation, renin release, and reduced urinary volume by activating the cAMP–PKA pathway in vascular endothelial cells, JG granular cells, and collecting duct cells, respectively, the molecular mechanisms underlying the natriuretic actions of EP_2 or EP_4 receptors are not so well investigated. It is hard to predict the overall effects of EP_2 or EP_4 agonists on fluid and BP regulation, although a beneficial role of the EP_2 and EP_4 receptors can be anticipated from studies in EP_2 receptor-deficient mice that had salt-sensitive hypertension, [39] and where EP_4 receptor blockade moderated salt and water excretion [28]. The cAMP/PKA pathway activated the amiloride-sensitive epithelial sodium channel (ENaC) and sodium-potassium adenosine triphosphatase by promoting the trafficking of these proteins to the cell membrane in collecting duct cells [41, 42]. This should be an antinatriuretic pathway. EP_2 and EP_4 receptor stimulation generally increases cAMP generation and signaling in the kidney. This would therefore be predicted to reduce sodium excretion and would apparently conflict with reports that EP_2 or EP_4 receptor blockade reduces sodium excretion [28, 39]; however, a solution may be found in our recent studies. We showed that the cAMP-dependent pathway decreased the phosphorylation of two key regulator kinases that enhance ENaC activity, Akt and serum- and glucocorticoid-regulated kinase-1. This should decrease ENaC activity and might thereby counteract the effect of cAMP on ENaC (unpublished data).

Conclusions and perspectives

Ample evidence suggests that inhibition of PGE_2 production is the major cause of the beneficial antipyretic and analgesic action of NSAIDs. However, the adverse side-effects of NSAIDs on fluid and BP regulation, including hypertension and edema formation, are also mediated predominantly by inhibition of PGE_2 production. Some of the pharmacological effects of PGE_2 may be dissected at the level of its receptors. This leads to the possibility that modulation of specific EP receptors by selective agonists could permit the use of NSAIDs to treat fever and pain with marginal adverse side-effects on fluid or BP regulation. Alternatively, this improved spectrum of actions might be achieved by selective blockade of specific EP receptors without the need for NSAIDs themselves.

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ORIGINAL ARTICLE

Use of xanthine oxidase inhibitor febuxostat inhibits renal interstitial inflammation and fibrosis in unilateral ureteral obstructive nephropathy

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Abstract
Background Renal interstitial fibrosis is the common pathway in progressive renal diseases, where oxidative stress promotes inflammation and macrophage infiltration. Febuxostat is a novel nonpurine xanthine oxidase (XO)-specific inhibitor for treating hyperuricemia. While some reports suggest a relationship between hyperuricemia and chronic kidney disease (CKD), the renoprotective mechanism of an XO inhibitor in CKD remains unknown. Recent reports have focused on XO as a source of oxidative stress.
Methods Here, we investigate the potential of febuxostat to reduce fibrogenic and inflammatory responses in an established interstitial fibrosis model—unilateral ureteric obstruction (UUO). Male Sprague–Dawley rats were divided into three groups: sham-operated group, vehicle-treated UUO group, and febuxostat-treated UUO group.
Results Treatment with febuxostat diminished XO activity in obstructed kidneys, and suppressed nitrotyrosine, a marker of oxidative stress. Consequently, febuxostat inhibited early proinflammatory cytokine expression, followed by a reduction of interstitial macrophage infiltration. In addition, febuxostat suppressed transforming growth factor-β messenger RNA expression, thereby ameliorating smooth muscle alpha actin and type I collagen expression.
Conclusion Our results provide evidence for the renoprotective action of febuxostat against the formation of interstitial fibrosis. A decrease in macrophage infiltration and interstitial fibrosis, along with a decrease of the oxidative stress marker, strongly suggests the existence of a causal relationship between them. Febuxostat may have therapeutic value in slowing or preventing interstitial fibrosis in patients with CKD.

Keywords Febuxostat · Ureteral obstruction · Tubulointerstitial injury

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Introduction

Accumulating evidence has shown a positive relationship between serum uric acid (UA) levels and cardiovascular mortality in patients with chronic kidney disease (CKD) [1, 2]. Xanthine oxidase/dehydrogenase (XOR) converts hypoxanthine and xanthine into xanthine and UA, respectively. The reduction in serum UA levels by administering the xanthine oxidase (XO) inhibitor allopurinol has been shown to slow the progression of renal dysfunction and decrease the risk of cardiovascular disease (CVD) in patients with CKD [3]. The renoprotective effects of an XO inhibitor were also shown in animal experimental models, including models using 5/6 nephrectomized rats [4] or diabetic mice [5]. This beneficial effect is thought to originate from a lowering of the plasma UA level, because UA itself has been shown to generate oxidative stress in adipocytes, vascular endothelial cells, and vascular smooth muscle cells [6–8].

Besides these hyperuricemia-related adverse effects, several studies have focused on XO as a source of oxidative stress. McCord [9] demonstrated that XOR functions in either a xanthine dehydrogenase (XDH) form, which transfers an electron to nicotinamide adenine dinucleotide (NAD^+) and generates NAD^+ hydrogen (NADH), or an

XO form, which transfers an electron to oxygen and generates oxidative stress. Calcium overload promotes conversion of the protein structure from XDH to XO. Due to this conversion, XO has been demonstrated to act as the major source of oxidative stress during ischemia reperfusion injury or acute renal-allograft rejection [10]. Angiotensin II-induced endothelial dysfunction also shows the significant role of XO in oxidative injury [11]. Renal interstitial fibrosis is one of the common histopathological features of progressive renal disease with diverse etiology. The unilateral ureteral obstruction (UUO) is a well-characterized experimental model of renal injury leading to tubulointerstitial fibrosis; however, the significant role of an increase in XO-dependent oxidative stress has been shown only after the release of obstructed ureter [12], and little information is available about the role of XO-induced oxidative stress in renal interstitial fibrosis.

The aim of this study was to investigate the role of XO activity on the progression of renal interstitial fibrosis in the UUO model. We used rats to minimize the influence of any UA-dependent action, based on the fact that the serum UA level in rodents is much lower than in humans. The effect of XO inhibition on renal interstitial fibrosis was tested by the administration of febuxostat, a newly developed XO inhibitor. In contrast to allopurinol, febuxostat does not inhibit other enzymes in the purine and pyrimidine metabolism pathway, and a sufficient dosage to inhibit XOR activity can be safely used even in subjects with damaged kidney function [13].

Materials and methods

Animals

Healthy male Sprague–Dawley rats (196–206 g body weight; Japan SLC Inc., Shizuoka, Japan) were maintained at the Institute of Experimental Animal Sciences of Osaka University Graduate School of Medicine, an accredited specific pathogen-free facility. The rats were housed in a constant-temperature room with a 12-h:12-h dark–light cycle, and fed food pellets, with ad libitum access to water.

Pretreatment and medication

In all animal experiments, the rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and handled in a humane manner in accordance with the guidelines of the Animal Committee of Osaka University. The rats were then assigned to one of three treatment groups: the UUO operation group (U); the UUO group receiving febuxostat (Teijin Pharma Ltd., Tokyo, Japan) medication (F); and the sham operation group (S).

The left ureter of each rat in groups U and F was ligated at the proximal end with 3-0 silk at two points and cut off at the middle. The sham operation consisted of a similar suprapubic incision and identification of the left ureter, but ligation of the ureter was not performed. All of the rats that underwent surgery were given intraperitoneal ampicillin.

Febuxostat (10 mg/kg/day) oral gavages were started at 24 h and 1 h before the operation, and continued every 24 h until sacrifice. This dosage was chosen as the maximal dosage that can be administered without forming the deposition of xanthine crystallizes in renal tubules [14]. The F group was administered 0.5% (w/v) methyl cellulose solution, given through a tube under anesthesia, whereas the respective U and S groups received solution without febuxostat.

The rats were sacrificed at days 1 ($n = 5$ for each group), 4 ($n = 8$ for each group), and 14 ($n = 3$ for each group) after surgery. A median incision was performed under anesthesia, blood samples were drawn from the aortic bifurcation, and urine samples were taken from the bladder (unaffected side) and obstructed renal pelvis (affected side). Subsequently, the obstructed kidneys in groups U and F, and the corresponding left kidneys in group S, were harvested. The kidneys were perfused with cold physiological salt solution and immediately decapsulated and cut into several pieces for the XO/XDH activity assay, nitro-oxidative stress assay, histological analysis, and RNA preparation.

XO/XDH activity assay

Measurement of XO and XDH activity in kidney tissue was based on the pterin-based assay [15]. In brief, approximately 100 mg of kidney tissues was homogenized in 1 mL assay buffer (50 mM K-phosphate, 1 mM ethylenediaminetetraacetic acid, 0.5% dimethyl sulfoxide, and protease inhibitor cocktail, pH 7.4). The supernatant (150 μL) was co-incubated with 50 μL pterin solution (final concentration of 50 μM) or pterin with methylene blue solution (final concentration of 50 μM) to assay XO or both XO and XDH activity, respectively. Before and after an 120-min incubation at 37°C, fluorometric assays were performed to calculate the production of isoxanthopterin. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Scientific Inc., Billerica, MA, USA).

Antibodies

Specific polyclonal antibodies for anti-smooth muscle α actin antibody (SMαA; 1:400, clone 1A4; Sigma-Aldrich, St. Louis, MO, USA), and, for macrophage staining, anti-rat CD68 (1:200, clone ED1, MCA341R; AbD Serotec, Kidlington, Oxfordfordshire, UK) were used in this study.

Morphology and immunohistochemical staining

Following fixation with 4% paraformaldehyde, the kidneys were processed to paraffin. Histological sections (2 μm) of the kidneys were used for periodic acid–Schiff (PAS) and Picrosirius red staining, or for immunohistochemical staining. Immunohistochemical staining was carried out by the standard avidin-biotinylated peroxidase complex method (Vectastain® ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA) with diaminobenzidine as the chromogen.

We scored and calculated the percentage of fibrosis, macrophage infiltration, and myofibroblasts in the interstitial area according to Sirius red-positive areas, the number of ED-1-positive cells in the interstitial space, and the percentage of SMαA staining-positive areas. All of the slides were highlighted on digitized images using a computer-aided manipulator (light microscopy; Nikon Eclipse 80i (Nikon, Tokyo, Japan), and pictures were taken with the Nikon ACT-1 ver. 2.63) Glomeruli and large vessels were excluded in the microscopic fields for image analysis. The scores of ten fields per each kidney section were averaged and used as the scores for the individual rats.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from whole kidneys using TRIzol® (Invitrogen, Carlsbad, CA, USA), and was reverse transcribed to complementary DNA (cDNA). Gene expression was measured by real-time quantitative PCR using an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA) with cDNA, SYBR® Green PCR Core Reagents (Invitrogen), and a set of primers. The primers for rat monocyte chemotactic protein-1 (MCP-1), interleukin-1 (IL-1), IL-12, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), SMαA, type I collagen, and 18S ribosome were as follows: MCP-1, 5′-atgcagttaatgcc ccactc-3′ (forward), 5′-ttccttattggggtcagcac-3′ (back); IL-1, 5′-gcctcgtgctgtctgacccat-3′ (forward), 5′-gggatccacactctccagc tgcag-3′ (back), 5′-ggcagttgggcaggtgacgt-3′ (back); TGF-β, 5′-tgcttcagctccacagagaa-3′ (forward), 5′-tggttgtagagggcaaggac-3′ (back); TNF-α, 5′-agatgtggaactggcagagg-3′ (forward), 5′-cccatttgggaacttctcct-3′ (back); SMαA, 5′-tccctggagaa-gagctacga-3′ (forward), 5′-tgaaagatggctggaagagg-3′ (back); type I collagen, 5′-ggccaggcagttctgattgg-3′ (forward), 5′-tcggct catgcgtggcctca-3′ (back); and 18S ribosome, 5′-cggctaccaca tccaaggaa-3′ (forward), 5′-agctggaattaccgcggc-3′ (back).

Nitrotyrosine enzyme-linked immunosorbent assay (ELISA)

Nitrotyrosine levels were quantified by ELISA using a nitrotyrosine ELISA kit (Northwest Life Science Specialties, LLC, Vancouver, WA, USA) according to the manufacturer's instructions. Nitrotyrosine standard or kidney homogenates were incubated with nitrotyrosine antibody in the microplate for 1 h; this was followed by incubation with streptavidin peroxidase for 1 h. The samples were incubated with tetramethylbenzidine substrate for 30 min, and the reaction was stopped by 2.0 mol/L citric acid. The formation of yellow product was measured at 450 nm.

Statistical analysis

All values are expressed as mean ± SE. Statistical analysis was evaluated using the Dunnett method by JMP version 9.0.0 (SAS Institute Inc., Cary, NC, USA), and $P < 0.05$ was considered to be statistically significant.

Results

Febuxostat suppressed renal XO and XDH activity

At day 1, renal tissue XO and XDH activity was not changed in the vehicle-treated obstructed kidneys compared with the sham-operated kidneys, but activity of both XO and XDH was induced on day 4. Treatment with febuxostat almost completely eliminated both XO and XDH activity on days 1 and 4 (Fig. 1a). Concomitant with the reduction in XO/XDH activity, febuxostat significantly reduced UA levels compared with vehicle treatment on day 4 (Fig. 1b). Compared with the sham-operated rats, the UUO rats exhibited impaired renal function, assessed by serum urea nitrogen (UN) and creatinine. Febuxostat had no significant effects on the elevated serum UN and creatinine levels (Fig. 1b).

Febuxostat ameliorated tubular damage and interstitial infiltration

Although both the vehicle- and febuxostat-treated obstructed kidneys showed ureteral dilatation to a similar extent, the vehicle-treated obstructed kidneys exhibited tubular damage, showing the rupture of the brush border and increased interstitial infiltration. Treatment with febuxostat ameliorated the rupture of the brush border and suppressed interstitial infiltration in the obstructed kidneys (Fig. 2a). Because we observed a protective effect of febuxostat on tubular damage, we examined the effect of febuxostat on proinflammatory cytokine expression in the obstructed kidneys. Real-time reverse transcriptase-PCR revealed that messenger RNA (mRNA) expression of MCP-1, TNF-α, and IL-1β was increased on day 1 in the obstructed kidneys. In contrast, febuxostat suppressed the

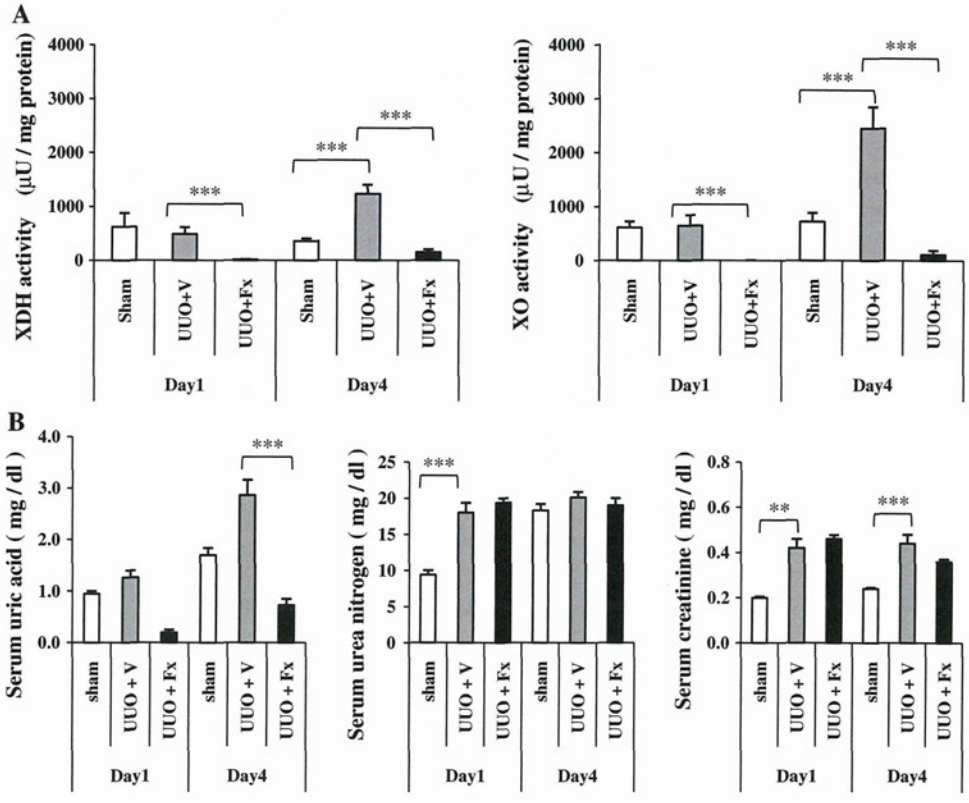


Fig. 1 Effects of UUO and treatment with febuxostat on renal XDH and XO activity, serum uric acid, urea nitrogen, and creatinine concentration. *sham* sham operation, *UUO + V* UUO operation without treatment, *UUO + Fx* UUO operation with febuxostat. Each parameter was assessed at days 1 and 4 after UUO. **a** XDH and XO activity in whole kidney extracts. $***P < 0.001$ compared with reference. **b** Serum concentration of uric acid, urea nitrogen, and creatinine. $**P < 0.01$, $***P < 0.001$ compared with reference

increase in these cytokine expressions (Fig. 2b). We next examined macrophage infiltration in the interstitium. The number of ED-1 positive macrophages was significantly increased in the interstitial area of the vehicle-treated obstructed kidneys on day 4. Parallel with the significant reduction of MCP-1 in the febuxostat-treated obstructed kidneys, febuxostat suppressed the infiltration of ED-1-positive macrophages, which was consistent with the observation from PAS staining (Fig. 2c, d). Concomitant with macrophage infiltration, the macrophage-derived cytokine IL-12β was increased in the vehicle-treated obstructed kidneys on day 4, but reversed in the febuxostat-treated kidneys (Fig. 2e).

Febuxostat inhibits interstitial fibrosis

We then examined the therapeutic effect of febuxostat on interstitial fibrosis in the obstructed kidneys. Similar to inflammatory cytokine expression, real-time reverse transcriptase-PCR demonstrated that TGF-β, SMαA, and type I collagen mRNA levels were increased in the obstructed kidneys compared with the sham-operated kidneys, while febuxostat suppressed the increase in mRNA expression on day 4 (Fig. 3a–c). Phenotypic alteration, assessed by immunohistological positive areas for SMαA on day 4, was augmented in the obstructed kidneys, but was mostly limited to the vessels in the febuxostat-treated kidneys (Fig. 3d, e). On day 14, Picrosirius red staining demonstrated that the febuxostat-treated obstructed kidneys exhibited significantly less interstitial fibrosis than the vehicle-treated kidneys (Fig. 4a, b).

Febuxostat inhibits oxidative stress

To investigate the therapeutic mechanism of febuxostat, we examined oxidative stress. On day 1, the nitrotyrosine concentration, a marker of nitro-oxidative stress, of the febuxostat-treated obstructed kidneys was lower than that of the vehicle-treated kidneys (Fig. 4c). Compared with the corresponding sham kidneys, the nitrotyrosine concentration of the obstructed kidneys did not change at day 1, but was reduced at day 4. (Day 1: sham kidneys, 2.74 ± 0.44 pmol/mg protein; obstructed kidneys, 2.58 ± 0.28 pmol/mg protein. Day 4: sham kidneys, 1.05 ± 0.26 pmol/mg protein; obstructed kidneys, 0.21 ± 0.04 pmol/mg protein, $P < 0.01$ vs. sham.)

Discussion

In the present study, we tested the hypothesis that febuxostat has therapeutic effects on tubulointerstitial injury in a rat UUO model, in which the plasma UA concentration is lower than in humans due to the presence of uricase. The obstructed kidneys exhibited increased proinflammatory and fibrogenic cytokines, thereby inducing macrophage

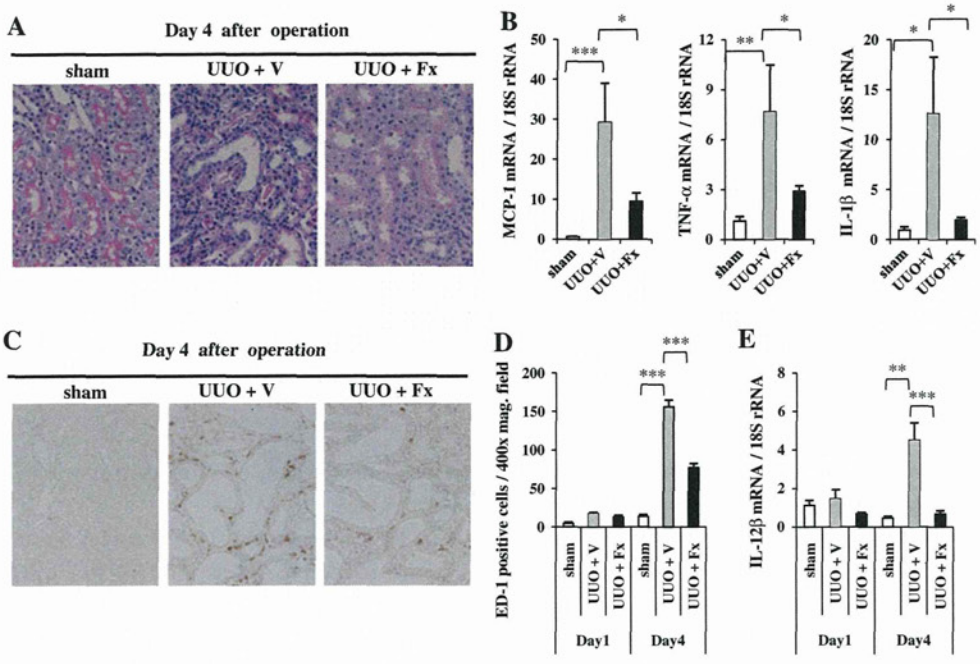
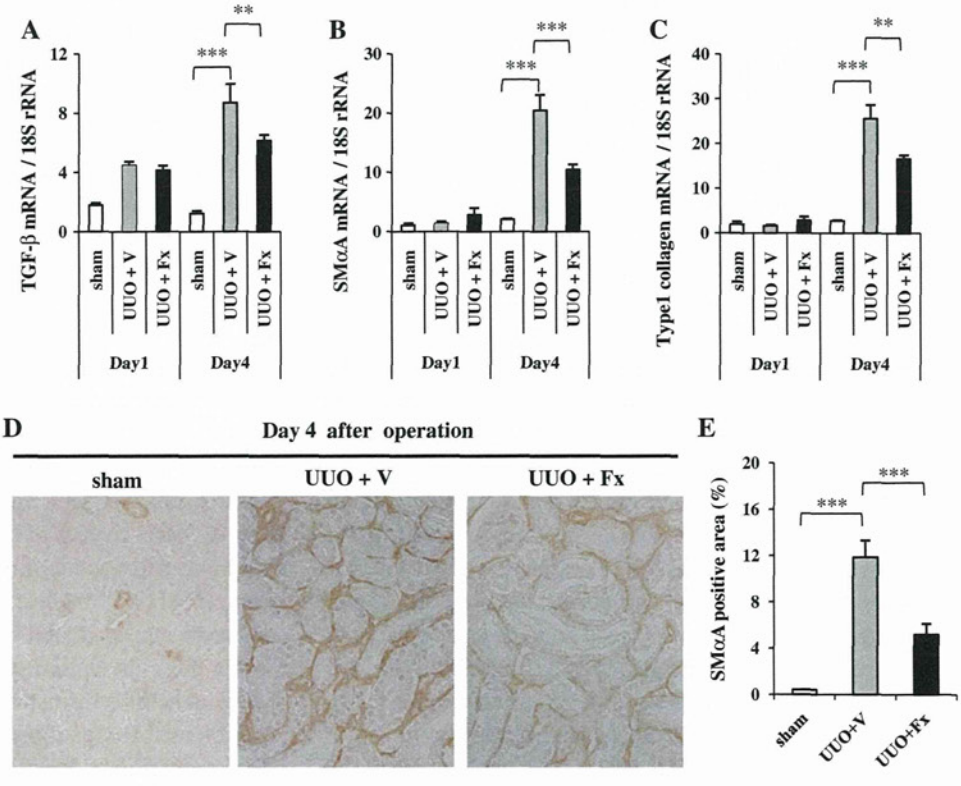


Fig. 2 Effects of UUO and treatment with febuxostat on renal morphology, inflammatory cytokines, and macrophage infiltration. **a** Periodic acid-Schiff staining at 4 days after operation. **b** mRNA expression levels of MCP-1, TNF-α, and IL-1β at day 1 after operation, as analyzed by real-time PCR. Results are expressed as relative expression against the expression of corresponding genes in sham. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with reference. Immunohistological staining for ED-1 (**c**) and the number of ED-1 positive cells in interstitial space per $\times 400$ magnification field (**d**) on day 4 after operation. $^{***}P < 0.001$ compared with reference. **e** mRNA expression levels of IL-12β at days 1 and 4 after operation, as analyzed by real-time PCR. Results are expressed as relative expression against the expression of corresponding genes in sham. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with reference

Fig. 3 Effects of UUO and treatment with febuxostat on renal fibrosis-related genes and SMαA. **a**–**c** mRNA expression levels of TGF-β, SMαA, and type I collagen at days 1 and 4 after operation, as analyzed by real-time PCR. Results are expressed as relative expression against the expression of corresponding genes in sham. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with reference. Immunohistological staining for SMαA (**d**), and the percentage of SMαA stained positive areas in interstitial space (**e**) on day 4 after operation. $^{***}P < 0.001$ compared with reference



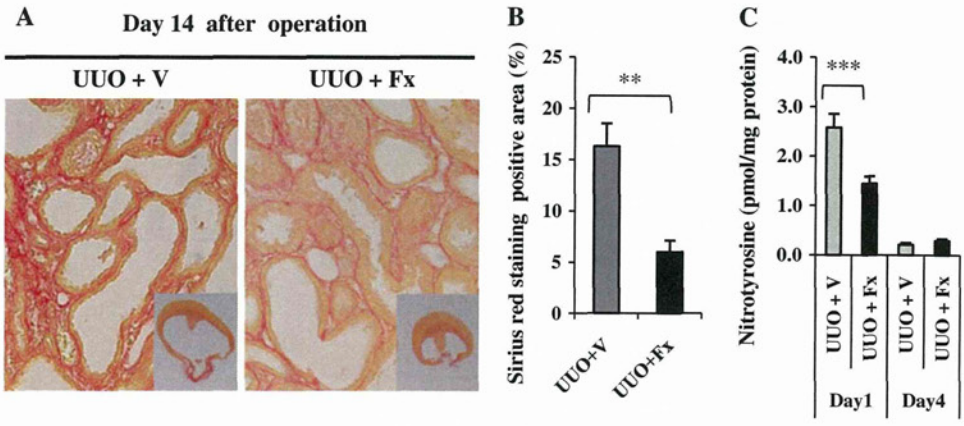


Fig. 4 Effects of UUO and treatment with febuxostat on renal fibrosis and nitrotyrosine concentration. Picrosirius red staining (**a**), and the percentage of Picrosirius red staining-positive fibrotic areas in interstitial space (**b**) on day 14 after UUO. $^{**}P < 0.01$ compared with reference. **c** Renal concentration of nitrotyrosine at days 1 and 4 after UUO was evaluated by ELISA. $^{***}P < 0.001$ compared with reference

infiltration and interstitial fibrosis. The administration of febuxostat ameliorated these manifestations by inhibiting the induction of proinflammatory and fibrogenic cytokines. Of interest is that febuxostat reduced nitro-oxidative stress, as assessed by nitrotyrosine, a marker of oxidative stress. We propose a new strategy for treating progressive kidney diseases using febuxostat as an anti-oxidant drug in addition to its effect on the reduction of UA.

We first demonstrated that febuxostat suppressed oxidative stress by assessing the nitrotyrosine level. Nitrotyrosine is a tyrosine nitration product mediated by reactive nitrogen species under proinflammatory conditions. Peroxynitrite anion is one of the most powerful reactive oxygen species that is produced by the reaction of nitric oxide (NO) and superoxide radicals, and is considered to be a marker of reactive nitrogen species induced by inducible nitric oxide synthase (iNOS), accompanied by oxidative stress [16]. We identified a lower production of nitrotyrosine in the febuxostat-obstructed kidneys; this may originate from both a complete blockade of XO activity and diminished induction of iNOS [7]. Several studies have focused on XO as a source of reactive oxygen species (ROS) production. XDH, which is unable to generate ROS, is converted to XO by cellular calcium overload [9]. XO can produce ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals [9, 17]. Because we showed that febuxostat diminished XO activity compared with the vehicle-treated obstructed kidneys, the reduction in XO activity might therefore have suppressed the production of nitrotyrosine in the febuxostat-treated obstructed kidneys. The concentration of nitro-tyrosine in sham kidney was identical or higher than that of UUO kidney. Although the precise mechanism of this unanticipated finding is not apparent, it may be explained by the decreased substrate of nitro-tyrosine in kidney. A previous report demonstrated that tyrosine escaped from damaged tissue to blood stream in a gut ischemia reperfusion model [18]. The formation of nitro-tyrosine depends on oxidative stress, NO and tyrosine. Therefore, the reduced concentration of tyrosine in damaged UUO kidney may be a possible cause of reduced production of nitro-tyrosine in UUO compared to sham kidney.

In the present study, we found a simultaneous increase in nitro-oxidative stress and MCP-1 mRNA induction on day 1, prior to the occurrence of macrophage infiltration on day 4. A previous report showed a positive interaction between ROS and macrophage infiltration. Oxidative stress promotes the expression of various inflammation-related molecules, including MCP-1, which, in turn, promotes inflammatory cell infiltration [19]. Together with the reduction in XO activity, febuxostat treatment clearly demonstrated anti-inflammatory effects, even at 1 day after ureteral obstruction. TNF-α and IL-1β mRNA expression were also suppressed in the febuxostat-treated obstructed kidneys on day 1. Furthermore, the inhibition of macrophage infiltration contributed to the reduction in the expression of IL-12β, one of the macrophage-derived cytokines, which otherwise promoted tubulointerstitial inflammation. Therefore, febuxostat may halt the vicious cycle involving tubules and macrophages.

We also demonstrated that febuxostat suppressed interstitial fibrosis. Febuxostat suppressed TGF-β, type I collagen and SMαA expression on day 4, resulting in significant interstitial fibrosis on day 14, as assessed by Picrosirius red staining. These results suggest that one aspect of the protective mechanism of febuxostat in a UUO kidney is the reduction of nitro-oxidative stress, which, in turn, might suppress the proinflammatory and fibrogenic cytokines, followed by a reduction in macrophage infiltration and tissue fibrosis. Interestingly, Landmesser et al. [11] demonstrated the crosstalk between angiotensin II signaling and conversion of XDH to XO in endothelial cells. Angiotensin II is known to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; Landmesser et al. showed that the oxidative stress created by NADPH oxidase activation promotes the conversion of the XDH form to the XO form, which generates further oxidative stress in endothelial cells. Angiotensin II signaling was also reported to contribute to the progression of fibrosis in UUO [20]. Angiotensin II and

TGF-β coordinately promote interstitial fibrosis [21]. Thus, the XOR system could possibly increase nitro-oxidative stress in the process of forming interstitial fibrosis. The amelioration of this XOR system may be a therapeutic target for the treatment of interstitial fibrosis.

The present study supports the current pathological concept that XO activity itself, rather than hyperuricemia, may play an important role in causing progressive tissue fibrosis. Several reports have suggested a UA-independent therapeutic effect of XO inhibitor. A clinical study by Ogino et al. [22] showed that benzbromarone lowered the level of UA, but had no effect on hemodynamic impairment in patients with chronic heart failure. A study by Sanchez-Lozada et al. [4] showed that an XO inhibitor provided a renoprotective effect in 5/6 nephrectomized rats without hyperuricemia [4]. Since XOR is expressed ubiquitously, the targeting of XO activity can be applied to a variety of tissue and disease conditions. Patients with CKD have been shown to have high oxidative stress [23]; a high protein conversion rate from XOR to XO is assumed to occur in those patients. The use of an XOR inhibitor in treating patients with CKD has been restricted due to the lack of appropriate agents, but there is now the novel agent febuxostat, which can be used effectively even in the presence of CKD. Further investigation is needed into the role of febuxostat in the progression of CKD. Although the reduction of UA itself may have a protective effect for patients with CKD, the UA-independent actions of an XOR inhibitor may play a significant role against the progression of CKD or CVD.

In conclusion, the results obtained from our non-hyperuricemic obstructed kidney model showed that XOR activity contributes to the progression of renal interstitial fibrosis by modulating oxidative stress and proinflammatory cell infiltration. Our observations support the current pathological concept that, in addition to hyperuricemia, an increase in XO activity itself may play an important role in the progression of tissue fibrosis. A novel XOR inhibitor, febuxostat, may be a therapeutic tool for treating progressive interstitial fibrosis.

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inappropriate selection of patients for randomization is the fact that mortality in the conventional arm was 1/42 = 2.38%, which is at least 7 times lower than that in the general population of hemodialysis (HD) patients in the United States. Many patients in the control group performed more frequent dialysis sessions and those in the more frequent group performed less frequent dialyses. The ultimate conclusion that was reached was that the frequent nocturnal dialysis study group had improvement in "control of hyperphosphatemia and hypertension but no benefit among other main secondary outcomes". Improvement of left ventricular mass was rejected as insignificant on the basis of a *P*-value of 0.09, which means a chance difference probability of 1 in 11 instead of 1 in 20. We wonder whether the Student's paired *t*-test, if performed in the patients in the 'frequent group', would show that a decrease of left ventricular mass from an average of 141 to 132 was in fact statistically significant. In all, this study committed a type II statistical error because of the evidently small number and inappropriate selection of subjects. On the basis of this study it absolutely cannot be accepted that frequent nocturnal HD is not better than conventional thrice-weekly HD. Interestingly, the front page of *Kidney International* egregiously highlights the rather misperceived notion that frequent nocturnal HD is of no benefit!

1. Rocco MV, Lockridge RS, Beck GJ *et al.* The effects of frequent nocturnal home hemodialysis: the Frequent Hemodialysis Network Nocturnal Trial. *Kidney Int* 2011; **80**: 1080–1091.
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The Authors Reply: Drs Misra and Twardowski[1] claim that subjects in the Frequent Hemodialysis Network (FHN) Nocturnal Trial[2] were inappropriately selected. All randomized subjects in the Nocturnal Trial had stage V chronic kidney disease and <20% of subjects had a urea clearance >3 ml/min. Although the mortality rate in both the FHN Daily and Nocturnal Trials was lower than that seen in the general hemodialysis population, this observation is common to randomized clinical trials and is likely due to the exclusion of subjects with a limited life expectancy who would not be influenced by the intervention. Less than 10% of subjects in the control arm performed dialysis ⩾4 times per week (Figure 2)[2] and 72% of subjects in the more frequent arm had ⩾4.8 treatments per week (Table 2).[2] In addition, the separation in the weekly dose of dialysis between arms was robust (total weekly standard Kt/V_{urea} of 5.03 ± 1.23 vs. 2.91 ± 0.86, $P<0.001$). While our findings for left ventricular mass (LVM) did not achieve statistical significance, the mean change and 95% confidence intervals for LVM were not materially different from the results of the FHN Daily Trial.[3] We provided several possible explanations for the non-significant effect of more frequent dialysis on LVM in our discussion,[2] as did Davenport in his commentary.[4] Finally, the summary statement on the front cover was not written by the authors but was provided by the *Kidney International* staff. We did not conclude that there is no benefit of nocturnal dialysis on LVM.

1. Misra M, Twardowski ZJ. Benefits of frequent nocturnal home hemodialysis. *Kidney Int* 2012; **82**: 114–115.
2. Rocco MV, Lockridge RS, Beck GJ *et al.* The effects of frequent nocturnal home hemodialysis: the Frequent Hemodialysis Network Nocturnal Trial. *Kidney Int* 2011; **80**: 1080–1091.
3. Chertow GM, Levin NW, Beck GJ *et al.* In-center hemodialysis six times per week versus three times per week. *N Engl J Med* 2010; **363**: 2287–2300.
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Implausible similarities in patient characteristics between two randomized controlled studies: a coincidence is unlikely

To the Editor: We have read the interesting article of the randomized controlled trial (RCT) by Goraya *et al.*[1] showing that 30-day dietary acid reduction with an aggressive intake of fruits and vegetables (F + V) has a renoprotective effect for stage 2 chronic kidney disease (CKD). Despite the small sample size, it clearly demonstrated the efficacy of F + V by both clinical and experimental surrogate markers. Its effect is almost the same with that of sodium bicarbonate, which had already been shown to attenuate the rate of decline of the glomerular filtration rate in a 5-year RCT by the same group of this study.[2] Thus, F + V without the increase in sodium intake may be more favorable than sodium bicarbonate for early CKD patients.

However, the characteristics of the participants with stage 2 CKD between these two RCTs are implausibly similar (Table 1). These similarities are impossible for two separate RCTs with at least a 5-year interval. Even if these studies were conducted in parallel without a detailed

Table 1 | Common patient characteristics quoted from the reports by Goraya *et al.*[1] and Mahajan *et al.*[2]

	Goraya *et al.*, CKD2 control (*n*=40)	Mahajan *et al.*, NaCl (*n*=40)	Goraya *et al.*, CKD2 and HCO_3 (*n*=40)	Mahajan *et al.*, $NaHCO_3$ (*n*=40)	Goraya *et al.*, CKD2 and F+V (*n*=40)	Mahajan *et al.*, placebo (*n*=40)
Males (%)	47.5	48	47.5	48	47.5	48
Black/white/Hispanic (%)	62.5/22.5/15.0	63/23/15	62.5/20.0/17.5	63/20/18	62.5/25.0/12.5	63/25/13
Age (years)	51.5 ± 8.3	51.5 ± 8.3	51.2 ± 8.2	51.2 ± 8.2	51.3 ± 8.5	51.3 ± 8.5
Systolic BP (mm Hg)	134.3 ± 8.3	152.6 ± 14.7	134.1 ± 5.8	155.3 ± 12.6	133.7 ± 8.6	155.2 ± 12.9
eGFR (ml/min)	75.6 ± 6.5	75.6 ± 6.5	75.3 ± 6.1	75.3 ± 6.1	75.6 ± 6.2	75.6 ± 6.2
Plasma total CO_2 (mmol/l)	26.0 ± 0.8	26.4 ± 0.8	25.9 ± 0.6	26.2 ± 0.7	25.9 ± 0.8	26.0 ± 0.9
PRAL (mmol/day)	59.3 ± 21.1	59.3 ± 21.1	64.3 ± 17.7	64.3 ± 17.7	60.4 ± 19.4	60.4 ± 19.4
8-h NAE (mEq)	24.6 ± 5.7	24.8 ± 6.4	24.8 ± 5.6	24.8 ± 5.6	24.6 ± 5.0	24.0 ± 5.6
Ualb (mg/g Cr)	413.6 ± 147.9	413.6 ± 147.9	419.3 ± 150.8	419.3 ± 150.8	422.2 ± 151.6	422.2 ± 151.6
UNAG (U/g Cr)	2.7 ± 0.4	2.6 ± 0.5	2.7 ± 0.4	2.7 ± 0.4	2.7 ± 0.7	2.7 ± 0.7
UET-1 (ng/g Cr)	5.5 ± 1.1	5.7 ± 0.8	5.7 ± 1.0	5.7 ± 1.0	5.5 ± 1.2	5.5 ± 1.2
Urine Na^+ excretion (mmol/g Cr)	71.6 ± 7.9	70.6 ± 10.2	70.9 ± 10.2	70.9 ± 10.2	73.0 ± 9.5	73.0 ± 9.5
Urine K^+ excretion (mmol/g Cr)	38.6 ± 5.5	38.2 ± 6.2	41.1 ± 6.1	41.1 ± 6.1	39.5 ± 6.6	39.5 ± 6.6

Abbreviations: BP, blood pressure; CKD, chronic kidney disease; Cr, creatinine; eGFR, estimated glomerular filtration rate; F+V, fruits and vegetables; NAE, net acid excretion; PRAL, potential renal acid load; Ualb, urine albumin-to-creatinine ratio; UET-1, urine endothein-1-to-creatinine ratio; UNAG, urine *N*-acetyl-β-D-glucosaminidase-to-creatinine ratio. Values are expressed as number, percentage, or mean ± SD as appropriate.

description, the 'NaCl' groups should not be considered as the control, and 'F + V' seems like placebo with 5-year observation.

The long-term follow-up of the MDRD study revealed the risk of the low-protein diet,[3] which has been believed to be good for CKD by both patients and health professionals for a long time. The interesting hypothesis of Goraya *et al.* could have been an alternative dietary intervention for CKD patients if proven appropriately. Disappointingly, the serious suspicion of faking research has made a mess of the series of their RCTs.

1. Goraya N, Simoni J, Jo C *et al.* Dietary acid reduction with fruits and vegetables or bicarbonate attenuates kidney injury in patients with a moderately reduced glomerular filtration rate due to hypertensive nephropathy. *Kidney Int* 2012; **81**: 86–93.
2. Mahajan A, Simoni J, Sheather SJ *et al.* Daily oral sodium bicarbonate preserves glomerular filtration rate by slowing its decline in early hypertensive nephropathy. *Kidney Int* 2010; **78**: 303–309.
3. Menon V, Kopple JD, Wang X *et al.* Effect of a very low-protein diet on outcomes: long-term follow-up of the Modification of Diet in Renal Disease (MDRD) Study. *Am J Kidney Dis* 2009; **53**: 208–217.

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The Authors Reply: We thank Dr Obi *et al.* for their comment[1] regarding similarities in subject groups in our two *Kidney International* publications[2,3] and their question of whether the two studies are truly randomized controlled studies given that some groups across the studies are very similar. Since 1999 we serially recruited subjects with hypertensive nephropathy and various estimated glomerular filtration rate (eGFR) levels to test the hypothesis that dietary acid reduction reduces acid retention,[4] reduces kidney injury,[2] and slows eGFR decline.[3] Subjects were recruited and serially entered into protocols designed to test these hypotheses. The first protocol randomized three sets of subjects with stage 2 eGFR, each of whom underwent three serial stages of studies to assess the effect of dietary acid reduction on acid retention (baseline and after 30 days), kidney injury (before and after 30 days), and, after a 30-day washout, the effect on eGFR after 5 years. Each subject was followed up in excess of 5 years, but in each case the follow-up began at recruitment and study entry. Specifically, they were not recruited as a group and then followed as a group at the same time. We initially submitted one manuscript to *Kidney International* that included the described three serial stages of study done on each subject to test all the three hypotheses. The Editors asked that we not test all the three hypotheses in a single manuscript, but to do so in separate manuscripts. We chose first to submit a manuscript detailing the main, randomized protocol in these stage 2 subjects that showed long-term (5-year) effects on eGFR[3] followed by separate submissions of the shorter-term portions of this protocol in many of the same subjects that examined the effects on acid retention[4] and, most recently, kidney injury.[2] Consequently, some subject groups that were randomized for the 5-year protocol to examine the effects of the interventions on eGFR were the same groups used to test the two remaining hypotheses as described but were not further randomized.

1. Obi Y, Hama H, Suzuki Y *et al.* Implausible similarities in patient characteristics between two randomized controlled studies: a coincidence is unlikely. *Kidney Int* 2012; **82**: 115–116.

AJKD
Original Investigation

Self-reported Sleep Duration and Prediction of Proteinuria: A Retrospective Cohort Study

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Background: Although multiple studies have shown that sleep duration is a predictor of cardiovascular diseases and mortality, few studies have reported an association between sleep duration and chronic kidney disease.

Study Design: Retrospective cohort study.

Setting & Participants: 6,834 employees of Osaka University aged 20-65 years who visited Osaka University Healthcare Center for their mandatory annual health examinations between April 2006 and March 2010 and did not have estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2, proteinuria, or treatment for self-reported kidney disease.

Predictor: Self-reported questionnaires about life style, including sleep duration, and blood and urine testing at the first examinations during the study period. An association between sleep duration and outcome was assessed using multivariate Poisson regression models adjusting for clinically relevant factors.

Outcome: Time to the development of proteinuria defined as 1+ or higher by dipstick test.

Results: Self-reported baseline sleep duration was 6.0 ± 0.9 hours, which reflected the mean sleep duration during a median of 2.5 (25th-75th percentile, 1.4-3.9) years of the observational period. Development of proteinuria was observed in 550 employees (8.0%). A multivariate Poisson regression model clarified that shorter sleep duration, especially 5 or fewer hours, was associated with the development of proteinuria in a stepwise fashion (vs 7 hours; incidence rate ratios of 1.07 [95% CI, 0.87-1.33; P = 0.5], 1.28 [95% CI, 1.00-1.62; P = 0.05], and 1.72 [95% CI, 1.16-2.53; P = 0.007] for 6, 5, and ≤4 hours, respectively), along with younger age, heavier current smoking, trace urinary protein by dipstick test, higher eGFR, higher serum hemoglobin A_{1c} level, and current treatment for heart disease. A stepwise association between shorter sleep duration and the development of proteinuria also was verified in 4,061 employees who did not work the night shift.

Limitations: Self-reported sleep duration might be biased. Results in a single center should be confirmed in the larger cohort including different occupations.

Conclusion: Short sleep duration, especially 5 or fewer hours, was a predictor of proteinuria.
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INDEX WORDS: Sleep duration; sleep deprivation; chronic kidney disease; proteinuria; Osaka University Healthcare Center.

Editorial, p. 325

Although sleep is one of the vital factors contributing to health, sleep duration has become shorter in modern societies in recent decades and the prevalence of sleep deprivation has increased, irrespective of socioeconomic status.[1] This trend is a major public health concern because multiple studies have shown that shorter sleep duration is associated with obesity,[2-4] hypertension,[5] diabetes,[6-8] cardiovascular diseases (CVDs),[9-12] and even death.[10,13,14] Most of these studies showed that the population with 5 or fewer hours of sleep duration was at significantly higher risk, whereas those with 7 hours of sleep duration were at lowest risk. More interestingly, a British cohort study showed that a decrease in sleep duration was associated significantly with cardiovascular mortality.[15] These results strongly suggest that sleep duration is a modifiable target of treatment modalities for CVD, such as smoking[16] and obesity.[17]

The modifiable lifestyle factors of smoking and obesity also have been studied as a potential target for the treatment of chronic kidney disease (CKD) charac-

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terized by proteinuria and decrease in glomerular filtration rate (GFR). Multiple studies have shown that smoking and obesity are risk factors for CKD[18] and that weight loss[19] and smoking cessation[20] are associated with improved renal outcomes. However, to our knowledge, no cohort study has reported an association between sleep duration and CKD.

The purpose of the present study was to investigate whether there is an association between the modifiable lifestyle factor of sleep duration and urinary protein, a key prognostic factor of CKD.[21] Results of the present study provide novel insight into the treatment strategy against CKD.

METHODS

Study Population

Candidate participants in the present retrospective cohort study were 10,445 employees of Osaka University aged 20-65 years who visited Osaka University Healthcare Center for their annual health examinations during the entry period between April 2006 and March 2010. In Japan, annual health examinations are mandatory for all employees by the Labor Standards Act.[22] Osaka University is located in a suburb of Osaka city, the third largest city in Japan, with a population of ~2.5 million. Of 9,697 (92.8%) employees with an estimated GFR (eGFR) ≥60 mL/min/1.73 m^2, negative or trace urinary protein by dipstick test, and no current treatment for self-reported kidney disease, we excluded 160 employees (1.5%) with missing baseline data, including 40 employees with missing baseline information about sleep duration (0.4%). After excluding 2,703 (25.9%) employees with a single visit during the observational period between April 2006 and March 2011, the present study finally included 6,834 employees (65.4%). Because of the retrospective nature of the present study, sample size was determined by the number of the employees of Osaka University who visited Osaka Healthcare Center during the entry period. The study protocol was approved by the ethics committee in Osaka University Healthcare Center.

Measurements

At the first visit during the entry period between April 2006 and March 2010, baseline data for the employees were measured. Demographic, physical, and laboratory data included age, sex, occupation, body mass index (BMI; body weight in kilograms divided by height in meters squared), and mean arterial pressure [MAP (mm Hg) = diastolic blood pressure + (systolic blood pressure − diastolic blood pressure)/3)], urinary protein and hematuria by dipstick test, hemoglobin A_{1c} level, and serum concentrations of creatinine, total cholesterol, triglycerides, and uric acid. Urinary protein and hematuria were examined using Uropaper III Eiken (Eiken Chemical, www.eiken.co.jp/en/index.html). The employees brought their first-void urine in the morning. Results of urine dipstick tests were interpreted by well-trained nurses and recorded as negative, trace, 1+, 2+, 3+, and 4+. Occupations were classified into clerical workers, academic researchers, engineers and technical assistants, health care workers in university hospitals, and other occupations.

Information about life style and current treatments for comorbid conditions was based exclusively on self-reported standard questionnaires, which all employees were required to fill out at every visit for their annual health examinations. Sleep duration was ascertained by the question "How long do you sleep?" There were 6 possible answers: 3 or fewer, 4, 5, 6, 7, or 8 or more hours. Excessive daytime somnolence was determined according to a positive answer to the question "Are you very sleepy during daytime?" The frequency of night shift was based on the question "How often do you work at night between 10:00 PM and 5:00 AM per month?", for which the possible responses were none, 1-5, 5-9, 10-14, or 15 or more nights. Smoking status was classified into non-, past, and current smokers, according to the question; "Do you smoke?", with possible answers I do not smoke, I quit smoking, or I smoke. If current smokers, the number of cigarettes smoked per day was ascertained by the question "How many cigarettes do you smoke per day?", with choices of 10 or fewer, 11-20, 21-40, 41-60, and 61 or more cigarettes. Frequency of drinking alcohol was asked by the question "How often do you drink per a week?", with responses of rarely, 1-3 days, 4-6 days, or every day. Diagnosis of comorbid conditions, including hypertension, diabetes, dyslipidemia, hyperuricemia, and heart disease, was made according to positive answers to the questions "Are you being treated for hypertension, diabetes, dyslipidemia, hyperuricemia, and/or heart disease?"

Mean sleep duration of each employee during the observational period was calculated based on all answers during the follow-up period, excluding baseline sleep duration. To calculate mean sleep duration during the observational period, 3 or fewer and 8 or more hours of sleep duration were regarded as 3 and 8 hours, respectively. Because of small numbers of employees with 3 or fewer hours of baseline sleep duration (n = 12 [0.2%]), employees with 3 or fewer and 4 hours of baseline sleep duration were categorized into a single group with 4 or fewer hours of baseline sleep duration. Similarly, employees with 8 or more hours of baseline sleep duration (n = 115 [1.7%]) were categorized into 7 or more hours of baseline sleep duration.

The outcome measure of the present study was the development of proteinuria, defined as urinary protein (≥1+) by dipstick test. The observational period was defined as time from the first visit during the entry period between April 2006 and March 2010 to (1) development of proteinuria or (2) last measurement of urinary protein before the end of March 2011, whichever came first. All date were retrieved from the electronic database in Osaka University Healthcare Center.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation or median and interquartile range, as appropriate, and categorical variables were expressed as number and proportion. Statistical significance was set at $P < 0.05$. For employees not immediately excluded on the basis of eGFR, proteinuria, or self-reported kidney disease, differences in baseline characteristics between employees included and excluded were compared using χ^2 test, t test, and Wilcoxon rank-sum test, as appropriate. Stepwise associations between baseline sleep duration and other clinical characteristics were compared using Cochran-Armitage test for trend and Jonckheere-Terpstra test for trend, as appropriate. Cumulative probability of proteinuria was calculated using the Kaplan-Meier method. Predictors of proteinuria were identified using log-rank test for trend and/or Poisson regression models adjusting for clinically relevant factors. The appropriateness of Poisson regression models was tested with a goodness-of-fit test using deviance statistic. Interactions between sleep duration and other covariates were assessed incorporating their interaction terms into multivariate models. Because night shift might affect sleep duration, as a sensitivity analysis, the association between sleep duration and the development of proteinuria was assessed in those who did not report working the night shift. Statistical analyses were performed using Stata, version 11.2 (Stata Corp, www.stata.com) and R, version 2.13.1 (The R Foundation for Statistical Computing, www.r-project.org/).

Table 1. Clinical Characteristics of Those Excluded and Included in the Study

		Included Employees				
			Night Shift			
	Excluded Employees	Total	No	Yes	P^a	P^b
No. of employees	2,703	6,834	4,061	2,773		
Demographic and physical data						
Age (y)	32 (28-38)	34 (29-42)	35 (29-44)	32 (28-38)	<0.001	<0.001
Men	1,405 (52.0)	3,445 (50.4)	1,723 (42.4)	1,722 (62.1)	0.2	<0.001
Occupation					<0.001	<0.001
Clerical workers	568 (21.0)	1,909 (27.9)	1,848 (45.5)	61 (2.2)		
Academic researchers	1,016 (37.6)	2,641 (38.6)	1,262 (31.1)	1,379 (49.7)		
Engineers and technical assistants	171 (6.3)	473 (6.9)	441 (10.9)	32 (1.2)		
Health care workers	922 (34.1)	1,736 (25.4)	447 (11.0)	1,289 (46.5)		
Other employees	26 (1.0)	75 (1.1)	63 (1.6)	12 (0.4)		
BMI (kg/m^2)	21.8 ± 3.2	21.8 ± 3.2	21.5 ± 3.2	22.2 ± 3.2	0.5	<0.001
MAP (mm Hg)	84 ± 12	85 ± 12	85 ± 13	85 ± 12	<0.001	0.3
Lifestyle data						
Smoking status					0.001	0.02
Nonsmokers	2,182 (80.7)	5,508 (80.6)	3,314 (81.6)	2,194 (79.1)		
Past smokers	193 (7.1)	589 (8.6)	327 (8.1)	262 (9,4)		
Current smokers						
1-10 cigarettes/d	147 (5.4)	259 (3.8)	139 (3.4)	120 (4.3)		
11-20 cigarettes/d	143 (5.3)	377 (5.5)	214 (5.3)	163 (5.9)		
≥21 cigarettes/d	38 (1.4)	101 (1.5)	67 (1.6)	34 (1.2)		
Alcohol consumption					0.03	<0.001
Rarely	1,425 (52.7)	3,625 (53.0)	2,234 (55.0)	1,391 (50.2)		
1-3 d/wk	807 (29.9)	1,880 (27.5)	1,057 (26.0)	823 (29.7)		
4-6 d/wk	211 (7.8)	560 (8.2)	304 (7.5)	256 (9.2)		
7 d/wk	260 (9.6)	769 (11.3)	466 (11.5)	303 (10.9)		
Sleep duration	6.0 ± 0.9	6.0 ± 0.9	6.1 ± 0.9	5.8 ± 0.8	0.5	<0.001
≥8 h	94 (3.5)	188 (2.8)	148 (3.6)	40 (1.4)		
7 h	684 (25.3)	1,682 (24.6)	1,170 (28.8)	512 (18.5)		
6 h	1,206 (44.6)	3,155 (46.2)	1,864 (45.9)	1,291 (46.6)		
5 h	597 (22.1)	1,543 (22.6)	749 (18.4)	794 (28.6)		
4 h	111 (4.1)	245 (3.6)	120 (3.0)	125 (4.5)		
≤3 h	11 (0.4)	21 (0.3)	10 (0.2)	11 (0.4)		
Excessive daytime somnolence	111 (4.1)	248 (3.6)	151 (3.7)	97 (3.5)	0.3	0.6
Laboratory data						
Urinary protein by dipstick test					<0.001	<0.001
Negative	2,522 (93.3)	6,535 (95.6)	3,923 (96.6)	2,612 (94.2)		
Trace	181 (6.7)	299 (4.4)	138 (3.4)	161 (5.8)		
Hematuria by dipstick test					0.001	<0.001
Negative	2,354 (87.1)	5,810 (85.0)	3,365 (82.9)	2,445 (88.2)		
Trace	50 (1.8)	134 (2.0)	84 (2.1)	50 (1.8)		
1+	186 (6.9)	642 (9.4)	450 (11.1)	192 (6.9)		
≥2+	113 (4.2)	248 (3.6)	162 (4.0)	86 (3.1)		
eGFR (mL/min/1.73 m^2)	91 ± 15	90 ± 15	90 ± 16	91 ± 15	0.009	0.008
Hemoglobin A_{1c} (%)	4.9 ± 0.4	4.9 ± 0.4	4.9 ± 0.4	4.9 ± 0.4	0.01	<0.001
Total cholesterol (mg/dL)	189 ± 32	192 ± 33	193 ± 33	190 ± 32	<0.001	<0.001
Triglycerides (mg/dL)	64 (45-96)	64 (45-95)	62 (44-91)	66 (46-101)	0.8	<0.001
Uric acid (mg/dL)	5.1 ± 1.4	5.1 ± 1.4	4.9 ± 1.4	5.3 ± 1.4	0.7	<0.001
Treatments for comorbid conditions						
Hypertension	52 (1.9)	145 (2.1)	100 (2.5)	45 (1.6)	0.5	0.02
Diabetes	9 (0.3)	38 (0.6)	25 (0.6)	13 (0.5)	0.2	0.4
Dyslipidemia	25 (0.9)	86 (1.3)	56 (1.4)	30 (1.1)	0.2	0.3
Hyperuricemia	15 (0.6)	42 (0.6)	27 (0.7)	15 (0.5)	0.7	0.5
Heart diseases	5 (0.2)	13 (0.2)	10 (0.2)	3 (0.1)	0.9	0.2

Note: Comprises 9,537 employees who did not have eGFR <60 mL/min/1.73 m^2, proteinuria, or treatment for self-reported kidney disease and were not missing baseline data. The 2,703 excluded employees were those who had a single visit during the study period. Continuous variables are shown as mean ± standard deviation or median (25th-75th percentile); categorical variables are given as number (percentage).

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure.

[a]Excluded employees versus included employees.

[b]Included employees without night shift versus included employees with night shift.