Policy options for economic evaluation

To draw significant policy implications from this economic evaluation, policy options from status quo need to be defined. Under the current SHC, the dipstick test to check proteinuria is mandatory, while serum Cr assay is not. However, some health insurers voluntarily provide serum Cr assay to participants in addition to SHC. We surveyed health insurers in five prefectures and found that 65.4% of them implement use of serum Cr assay. Also, we analysed the Japan Tokutei-Kenshin CKD Cohort 2008 and found that 57.3% of participants underwent use of serum Cr assay. Therefore, we define the status quo regarding screening test for CKD as 40% of insurers implementing dipstick test only and 60% implementing dipstick test and serum Cr assay.

Then we evaluate two policy options in this study: 'Policy 1: Requiring serum Cr assay', and 'Policy 2: Requiring serum Cr assay and abandoning dipstick test'. Policy 1 means mandating use of serum Cr assay in addition to the currently used dipstick test, so that 100% of insurers implement both dipstick test and serum Cr assay if policy 1 is taken. Policy 2 is considered based on two recent health policy contexts. One is the discussion aroused during the development of SHC in which requiring serum Cr assay only and abandoning dipstick test used in the former occupational health checkup scheme attracted substantial support. It is expected that such a policy option will be proposed in the revision of SHC. Another relates to the change in diagnosis criterion of diabetes [35], in which a blood test to check the level of haemoglobin A1c instead of a dipstick test to check urinary sugar level has become pivotal. Implementing dipstick test for checking proteinuria only bears scrutiny from the viewpoint of economic evaluation. We assume that 100% of insurers would stop providing dipstick test if policy 2 is adopted.

We calculate incremental cost-effectiveness ratios (ICERs) for these two policy options using our economic model. ICER is a primary endpoint of cost-effectiveness analysis, which is defined as follows:

$$\mathrm{ICER} = \frac{\text{Incremental cost}}{\text{Incremental effectiveness}} = \frac{\mathrm{Cost}_{\text{New policy}} - \mathrm{Cost}_{\text{Status quo}}}{\mathrm{Effectiveness}_{\text{New policy}} - \mathrm{Effectiveness}_{\text{Status quo}}}$$

This means the additional cost required to gain one more QALY under new policy.

Sensitivity analysis

Economic modelling is fundamentally an accumulation of assumptions adopted from diverse sources. Therefore, it is imperative to appraise the stability of the model. We perform one-way sensitivity analyses for our model assumptions. Assumed probabilities about the participant cohort, the decision tree and the Markov model are changed by ±50%. Reductions of transition probabilities brought about by treatment are also changed by ±50%. Utility weights for quality of life adjustments are changed by ±20%. Costs are changed by ±50%. Discount rate is changed from 0% to 5%. We also changed our assumption about status quo that 40% of insurers implement dipstick test only and 60% implement dipstick test and serum Cr assay by ±50% as well.

Results

Model estimators

Table 2 presents the model estimators. Under the do-nothing scenario, no patient is screened, with average cost of renal disease care per person of ¥2,125,490 (US $23,617) during average survival of 16.11639 QALY. When (a) dipstick test to check proteinuria only is applied, 832 patients out of 100,000 participants are screened, with additional cost of ¥7,288 (US $81) per person compared with the do-nothing scenario, for additional survival of 0.00639 QALY (2.332 quality-adjusted life days). When (b) serum Cr assay only is applied, 3,448 patients are screened with additional cost of ¥390,002 (US $4,333) per person compared with the do-nothing scenario, for additional survival of 0.04801 QALY (17.523 quality-adjusted life days). When (c) dipstick test and serum Cr assay are applied, 3,898 patients are screened with additional cost of ¥395,655 (US $4,396) per person compared with the do-nothing scenario, for additional survival of 0.04804 QALY (17.535 quality-adjusted life days).

Model estimators of ICERs were calculated as ¥1,139,399/QALY (US $12,660/QALY) for (a) dipstick test only, ¥8,122,492/QALY (US $90,250/QALY) for (b) serum Cr assay only and ¥8,235,431/QALY (US $91,505/QALY) for (c) dipstick test and serum Cr assay.

Cost-effectiveness

Table 3 presents the results of cost-effectiveness analysis. Regarding the status quo that 40% of insurers implement dipstick test only and 60% implement dipstick test and serum Cr assay, 2,837 patients out of 100,000 participants are screened, with average cost of screening and renal disease care per person of ¥2,365,798 (US $212,922) during average survival of 16.14777 QALY. Taking policy 1 that 40% of insurers currently using dipstick test only start use of serum Cr assay screens more patients (3,898).

Table 2 Model estimators

	No. of patients per 100,000 participants	Cost (¥)	Incremental cost (¥)	Effectiveness (QALY)	Incremental effectiveness (QALY)	Incremental cost-effectiveness ratio (¥/QALY)
Do-nothing	0	2,125,490		16.11639		
(a) Dipstick test only	832	2,132,778	7,288	16.12278	0.00639	1,139,399
(b) Serum Cr assay only	3,448	2,515,492	390,002	16.16440	0.04801	8,122,492
(c) Dipstick test and serum Cr assay	3,898	2,521,145	395,655	16.16443	0.04804	8,235,431

Table 3 Results of cost-effectiveness analysis

	No. of patients per 100,000 participants	Cost (¥)	Incremental cost (¥)	Effectiveness (QALY)	Incremental effectiveness (QALY)	Incremental cost-effectiveness ratio (¥/QALY)
Status quo	2,837	2,365,798		16.14777		
Policy 1: requiring serum Cr assay	3,898	2,521,145	155,347	16.16443	0.01666	9,325,663
Policy 2: requiring serum Cr assay and abandoning dipstick test	3,448	2,515,492	149,694	16.16440	0.01663	9,001,414

It costs more, but it gains more. Its incremental cost is ¥155,347 (US $1,726), and its incremental effectiveness is 0.01666 QALY (6.081 quality-adjusted life days), resulting in ICER of ¥9,325,663/QALY (US $103,618/QALY). Taking policy 2 that 40% of insurers currently using dipstick test only start use of serum Cr assay and abandon dipstick test screens more patients (3,448) compared with the status quo as well. It also costs more, but it gains more. Its incremental cost is ¥149,694 (US $1,663), and its incremental effectiveness is 0.01663 QALY (6.070 quality-adjusted life days), resulting in ICER of ¥9,001,414/QALY (US $100,016/QALY).

Stability of cost-effectiveness

One-way sensitivity analyses produce similar results not only between policy 1 and policy 2 but also among three model estimators of ICER. Therefore, we present a tornado diagram of policy 1 as an example in Fig. 2. Ten variables with large change of ICER are depicted. A threshold to judge cost-effectiveness is also drawn, which is according to World Health Organization's (WHO) recommendation, being three times gross domestic product (GDP) per capita [36]. Its value is ¥11.5 million/QALY (US $128 thousand/QALY) gain in 2009 in Japan.

The effectiveness of CKD treatment to delay progression to ESRD is found to be the most sensitive. Decreasing the effect by 50% increases ICER to ¥16,280,537/QALY (US $180,895/QALY). The effectiveness of CKD treatment to prevent stroke is also found to be the 10th largest change of ICER, but its range is limited.

The cost of treatment for stage 5 CKD is found to be the second most sensitive. Increasing the cost by 50% increases ICER to ¥14,404,335/QALY (US $160,048/QALY). The cost of ESRD treatment is found to be the fifth largest change, and the change is in the opposite direction; decreasing this increases ICER. Another cost item depicted is the cost of treatment for stage 3 CKD, which is found to be the sixth largest change.

The discount rate is found to be the third most sensitive. Discounting at a rate of 5% makes ICER ¥11,373,185/QALY (US $126,369/QALY). Since policy 1 can screen CKD patients without proteinuria by use of serum Cr assay, the prognosis of non-proteinuric stage 5 CKD without treatment is found sensitive as the fourth and the seventh largest change. The eighth largest change depicted relates to the prevalence of CKD in participating population, i.e. stage 2 CKD without proteinuria. The ninth largest change is utility weight for ESRD.

Taking the threshold to judge cost-effectiveness, one-way sensitivity analyses alter the interpretation of the results for only three variables: reductions of transition probabilities from (1) screened and/or examined to (2) ESRD with the treatment of CKD; cost of treatment for stage 5 CKD; and transition probability from (1) screened and/or examined to (2) ESRD with no treatment by initial renal function for stage 5 CKD without proteinuria.

Discussion

We conduct a cost-effectiveness analysis of CKD screening test in SHC. Facing the scheduled revision of mandatory test items, we appraise two possible policy options compared with the status quo that 40% of insurers implement dipstick test to check proteinuria only and 60% implement

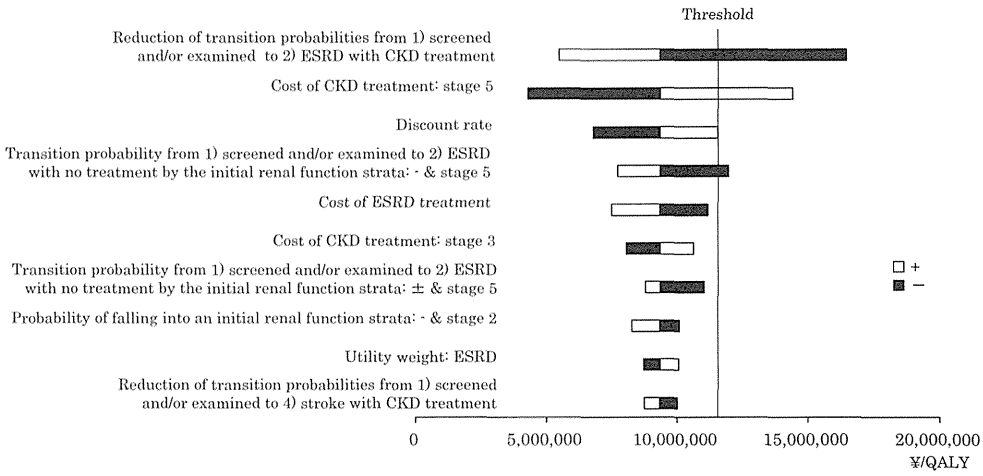

Fig. 2 Tornado diagram of policy 1. This tornado diagram shows ten variables which are found to be sensitive to the change in assumptions. Ten variables are presented, ordered according to the size of the change of ICER from top to bottom. The change of ICERs is represented by *white bars* when increasing the variable or by *black bars* when decreasing the variable from base-case value. The threshold to judge cost-effectiveness is 3 × GDP per capita (¥11.5 million/QALY gain)

dipstick test and serum Cr assay. Policy 1 is to mandate serum Cr assay in addition to the current dipstick test, so that 100% of insurers implement both dipstick test and serum Cr assay. Policy 2 is to mandate serum Cr assay and abandon dipstick test, so that 100% of insurers would stop providing dipstick test and switch to serum Cr assay. Our base-case analysis suggests that both policy options cost more and gain more. Estimated ICERs are ¥9,325,663/QALY (US $103,618/QALY) for policy 1 and ¥9,001,414/QALY (US $100,016/QALY) for policy 2.

To interpret these ICERs, there is no established value of social willingness to pay for one QALY gain in public health programmes such as mass screening in Japan, although some suggest ¥5 million/QALY (US $56 thousand/QALY) for an innovative medical intervention [37]. We follow WHO recommendation in this study, which is three times GDP per capita [36]. Its value is ¥11.5 million/QALY (US $128 thousand/QALY) gain in 2009 in Japan. Given this threshold, both policy 1 and policy 2 are judged as cost-effective. Therefore, mandating serum Cr assay in SHC can be justifiable as an efficient allocation of finite resources for health. Between policy 1 and policy 2, the ICER of policy 2 is slightly more favourable than that of policy 1, while 450 more patients out of 100,000 participants are screened by adopting policy 1. If secondary prevention of CKD is emphasised as a policy objective in addition to efficiency, policy 1 is an acceptable option as well as policy 2.

Our model estimators have a policy implication, although estimated ICERs do not directly depict any marginal change in society. The ICER of (a) dipstick test only compared with the do-nothing scenario, ¥1,139,399/QALY (US $12,660/QALY), is remarkably favourable. This implies that mass screening with dipstick test only is cost-effective compared with abolishment of mass screening for kidney diseases altogether. Therefore, continuing the current policy, i.e. mandatory dipstick test, could be justifiable as an efficient resource allocation.

This contrasts with the reported cost-ineffectiveness of annual mass screening for adults using dipstick test to check proteinuria in the USA [12], although direct comparison cannot be made between the results of economic evaluations under different health systems. The difference could be attributable to the difference in the prevalence of proteinuria among screened population, with 5.450% being used in our model based on the Japan Tokutei-Kenshin CKD Cohort 2008, while 0.19% is assumed in the US study. Such epidemiological differences are known in terms of not only quantity but also in quality [7]. The prevalence of glomerulonephritis, especially IgA nephropathy, is higher in Asian countries including Japan compared with Western countries [10]. Also, the prevalence of renovascular disease such as ischaemic nephropathy, with which patients are often non-proteinuric until advanced stages of CKD, is lower in Asian countries [38]. The inclusion of heart attack and stroke into our model, which are excluded in the US model [12], may have also made the ICER more favourable.

There is a report of cost-ineffectiveness of population-based screening for CKD with serum Cr assay from Canada [39]. This Canadian model can be compared with our model estimators of (b) serum Cr only compared with the do-nothing scenario. Their health outcomes gain or incremental effectiveness is 0.0044 QALY, which is smaller than ours, 0.04801 QALY, while their incremental cost is C $463 (US $441, using US $1 = C $1.05), which is also smaller than ours, ¥390,002 (US $4,333). These differences probably reflect the difference in the prevalence of CKD between Canada and Japan. Regarding the efficiency of screening programme, our model estimator of ICER, ¥8,122,492/QALY (US $90,250/QALY), is slightly more favourable than that of Canada, C $104,900/QALY (US $99,905/QALY). However, the contradictory conclusion regarding cost-effectiveness is not due to this difference but rather the threshold taken. The Canadian study adopts lower value such

as C $20,000 to C $50,000/QALY (US $19,048 to US $47,619/QALY) following local practice [40].

Our sensitivity analysis suggests instability of the results in only three variables, so our findings are robust to a certain extent. The most sensitive variable is the effectiveness of CKD treatment delaying progression to ESRD: 42.1% reduction is adopted in our economic model according to the unique clinical evidence from Japan, whose agent is angiotensin-converting enzyme inhibitor. It is marginally larger than comparative values reported from Western countries. Reductions in the rate of GFR decline are 35.9% by Agodoa et al. [41], 39.8% by The GISEN Group [42] and 22.5% by Ruggenenti et al. [43]. However, we think our assumption of base-case value is reasonable in two accounts: in light of the indication of angiotensin receptor blockers [17], whose use is more tolerated than angiotensin-converting enzyme inhibitors [44], and the higher prevalence of glomerulonephritis including IgA nephropathy, being a primary renal disease for ESRD, in Japan [10], for which the effect of early treatment such as renin–angiotensin system (RAS) inhibition, an immunosuppression, reduces risk of ESRD by 60% [45].

In regards to the other sensitive variables, we think the prognosis of non-proteinuric stage 5 CKD without treatment does not greatly undermine our findings of base-case analysis, since the value is calculated from extended follow-up of an established database [18]. Uncertainty of the base-case value should be much less than the analysed ±50%. On the other hand, the cost of treatment for stage 5 CKD relates to one of the weaknesses of this study, as discussed in the following.

There are weaknesses in this study. The most significant one is that our economic model depicts the prognosis of CKD by initial renal function stratum. This approach is taken because of the limitation of epidemiological data, and it has little difficulty in estimating outcomes in terms of survival. However, it becomes problematic when it comes to costing. For example, a patient initially screened as stage 1 CKD stays at (1) screened and/or examined before transiting to the following health states such as (2) ESRD. This means that a patient skips over stage 2 CKD to 5 CKD before progressing to ESRD. To estimate the cost for this health state, the diversity of patients in terms of progression of the CKD stages should be taken into account. Our expert committee has developed treatment models to understand this problem. This type of uncertainty is larger in stage 1 CKD and smaller in stage 5 CKD, but the cost of stages 1–4 CKD are not found to be so sensitive in our sensitivity analysis. Also, we think that uncertainty of the cost of stage 5 CKD, the second most sensitive variable, is less than the analysed ±50%, and our findings based on the base-case analysis are plausible. The problem also affects quality of life adjustment, which tends to produce larger QALY outcomes.

Table 4 Recommendation of the Japanese Society of Nephrology Task Force for the validation of urine examination as a universal screening

Mandate use of serum Cr assay in addition to the current dipstick test in the next revision of SHC

Other weaknesses include our assumption of 100% adherence to treatment and so on. However, the most significant strength of this study is that our economic model depends totally on evidence from Japan only, which could justify our simplification in modelling on data availability basis. There is an opportunity for further refinement of our economic model, because a large-scale field trial evaluating the effect of multifactorial treatment including lifestyle modification for early-stage CKD [46] is ongoing in Japan, which will enable us to model progression of CKD with more rigorous clinical evidence [47].

In conclusion, we, the Japanese Society of Nephrology Task Force for the Validation of Urine Examination as a Universal Screening, recommend to mandate use of serum Cr assay in addition to the current dipstick test in the next revision of SHC, from the viewpoint of value for money and the importance of secondary prevention (Table 4). We think that continuation of current policy, in which dipstick test only is mandatory, is still a sensible policy option. Development of adequate Specific Counselling Guidance for screened participants is also recommended.

Whereas the primary objective of this study is to appraise policy options in Japanese context, it also demonstrates that good value for money can be expected from mass screening with dipstick test to check proteinuria in population with high prevalence; that is, a population strategy could be adopted for control of CKD. However, caution is needed when extrapolating this conclusion, since the scope of costing of our economic model does not cover the initial cost of launching mass screening. The model here is based on currently running SHC. The practice of annual mass screening for adults in Japan is quite exceptional, while such universal programmes are rarely found in other countries [48].

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Conflict of interest The authors have declared that no conflicts of interest exist.

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Urinary FSP1 Is a Biomarker of Crescentic GN

Masayuki Iwano,*† Yukinari Yamaguchi,† Takaaki Iwamoto,‡ Kimihiko Nakatani,† Masaru Matsui,† Atsushi Kubo,† Yasuhiro Akai,† Toshio Mori,‡ and Yoshihiko Saito†

*Division of Nephrology, Department of General Medicine, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan; and †Department of First Internal Medicine and ‡Radioisotope Research Center, Nara Medical University, Kashihara, Nara 634-8522, Japan

ABSTRACT

Fibroblast-specific protein 1 (FSP1)–expressing cells accumulate in damaged kidneys, but whether urinary FSP1 could serve as a biomarker of active renal injury is unknown. We measured urinary FSP1 in 147 patients with various types of glomerular disease using ELISA. Patients with crescentic GN, with or without antinuclear cytoplasmic antibody–associated GN, exhibited elevated levels of urinary FSP1. This assay had a sensitivity of 91.7% and a specificity of 90.2% for crescentic GN in this sample of patients. Moreover, we found that urinary FSP1 became undetectable after successful treatment, suggesting the possible use of FSP1 levels to monitor disease activity over time. Urinary FSP1 levels correlated positively with the number of FSP1-positive glomerular cells, predominantly podocytes and cellular crescents, the likely source of urinary FSP1. Even in patients without crescent formation, patients with high levels of urinary FSP1 had large numbers of FSP1-positive podocytes. Taken together, these data suggest the potential use of urinary FSP1 to screen for active and ongoing glomerular damage, such as the formation of cellular crescents.

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Crescentic GN is a particularly aggressive type of kidney disease in which glomerular injury causes rapidly progressive GN.[1,2] Strong immunosuppressive therapy should be administered as early as possible in order to prevent irreversible kidney scarring.[3] The widespread use of assays for antinuclear cytoplasmic antibody (ANCA) has facilitated the clinical diagnosis of pauci-immune crescentic GN.[4,5] However, there have been few studies of biomarkers that could potentially serve to identify all forms of crescentic GN.

Fibroblast-specific protein 1 (FSP1) is one of the S100 calcium-binding proteins, a family of secreted and cytosolic proteins involved in a variety of biologic processes.[6–8] A large number of FSP1-expressing cells ($FSP1^+$ cells) accumulate in kidneys showing active renal damage.[9–11] In this study, we hypothesize that FSP1 secreted from $FSP1^+$ cells in the kidney should be detectable in urine samples. To test that idea and to clarify the significance of urinary FSP1 as a biomarker of active glomerular damage, we established two monoclonal antibodies for human FSP1 and developed a method for measuring urinary FSP1 levels using a sandwich-type ELISA. We then used that assay to assess urinary FSP1 excretion in cases of human GN.

Urinary FSP1 levels were measured in 147 patients with various types of glomerular disease (Figure 1A). In patients with ANCA-associated GN, urinary FSP1 levels were significantly higher (median, 3.71 μg/g of creatinine [first quartile, third quartile, 0.71, 5.07 μg/g of creatinine]) than in patients with IgA nephropathy (0.0 μg/g of creatinine [0.0, 0.98 μg/g of creatinine]; $P<0.001$), minimal-change nephrotic syndrome (0.0 μg/g of creatinine [0.0, 0.87 μg/g of creatinine]; $P<0.0001$), or membranous nephropathy (0.0 μg/g of creatinine [0.0, 0.0 μg/g of creatinine]; $P<0.0001$). Urinary FSP1 was not detectable in any of the healthy volunteers. In 56 patients with IgA nephropathy, the percentages of glomeruli showing cellular crescents, fibrocellular crescents, global sclerosis, and segmental sclerosis correlated positively with urinary FSP1 levels (Supplemental Table 1). A high level of urinary FSP1 (5.21 μg/g of creatinine) was also observed in one patient with FSGS showing a cellular variant.

Urinary FSP1 levels did not differ between patients with primarily cellular crescents or fibrocellular crescents, but urinary FSP1 was undetectable in five patients with fibrous crescents (Supplemental Figure 1). In five patients with IgA nephropathy and three patients with lupus nephritis, cellular or fibrocellular crescents were identified in more than 20% of glomeruli (20% crescent

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Correspondence: Dr. Masayuki Iwano, Division of Nephrology, Department of General Medicine, Faculty of Medical Sciences, University of Fukui, 23-3 Matsuokashimoaizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193 Japan. Email: miwano@u-fukui.ac.jp

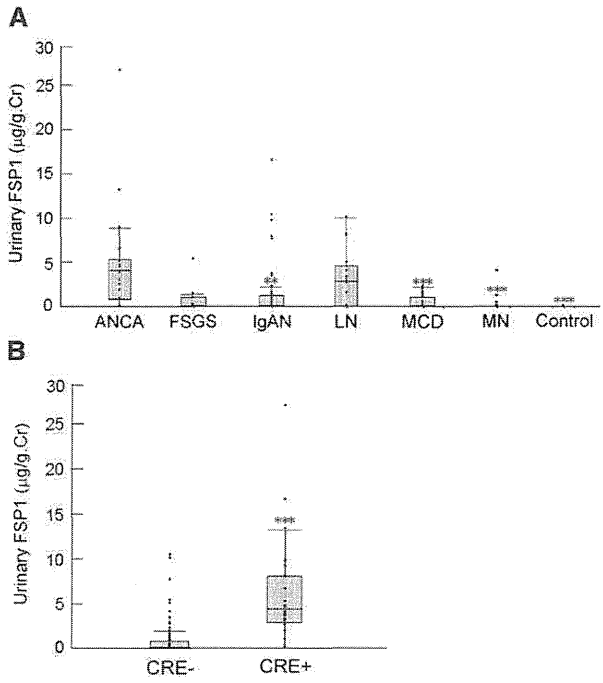

Figure 1. Elevation of urinary FSP1 in patients with ANCA nephritis and crescent formation. (A) Urinary FSP1 levels were measured in patients with ANCA-associated glomerulonephritis (ANCA), FSGS, IgA nephropathy (IgAN), lupus nephritis (LN), minimal-change nephrotic syndrome (MCD), or membranous nephropathy (MN). Urinary FSP1 levels were elevated in patients with ANCA and were undetectable in healthy volunteers (control). $^{**}P<0.001$ and $^{***}P<0.0001$ versus ANCA. Cr, creatinine. (B) Urinary FSP1 levels were significantly higher in patients with 20% crescent formation (CRE+) than those without it (CRE−).

formation). Given that urinary FSP1 levels were markedly elevated in these eight patients (5.93 μg/g of creatinine [3.45, 9.34 μg/g of creatinine), we divided the 147 study participants into two groups (with or without 20% crescent formation) and compared urinary FSP1 between these two groups. We found that urinary FSP1 levels were significantly higher in patients with 20% crescent formation than in those without it (4.48 μg/g of creatinine [2.91, 8.03 μg/g of creatinine] versus 0 μg/g of creatinine [0, 0.72 μg/g of creatinine]; $P<0.0001$) (Figure 1B).

To assess the diagnostic value of urinary FSP1 as a novel marker for crescent formation, we used receiver-operating characteristic curve analysis to determine a cut-off level for urinary FSP1 (Supplemental Figure 2) and made a 2×2 table. At FSP1 levels greater than 1.75 μg/g of creatinine, the assay had 90.2% specificity and 91.7% sensitivity for diagnosis in patients with 20% crescent formation. The positive and negative predictive values were 64.7% and 98.2%, respectively. We can also specifically select patients with 15% crescent formation by changing the FSP1 cut-off levels to greater than 5.0 μg/g of creatinine because both the specificity and the positive predictive value increased (to 98.3% and 86.7%, respectively), although the sensitivity decreased (to 43.3%).

The proteinuria level is a classic and valuable prognostic marker of CKD. However, proteinuria is not helpful for determining whether glomerular damage is ongoing. There have been a few studies of potential biomarkers for crescentic GN. Kanno and colleagues[12] showed that levels of urinary sediment podocalyxin are elevated in children with cellular crescents. Levels of urinary macrophage migration inhibitory factor and matrix metalloproteinase activity are reportedly higher in patients with crescentic GN and ANCA-associated GN than in healthy controls,[13,14] but the levels are not significantly higher than in patients with other glomerular diseases. By contrast, urinary FSP1 levels strongly correlated with the percentage of glomeruli showing cellular or fibrocellular crescent formation in patients with crescent formation, irrespective of the specific glomerular disease (Supplemental Figure 3). In addition, we confirmed a superiority of urinary FSP1 over other existing screening tests (C-reactive protein and serum creatinine) in diagnosing ANCA-negative crescentic GN (Supplemental Figure 4). These results suggest that urinary FSP1 may be useful for the diagnosis and management of all forms of crescentic GN.

We measured urinary FSP1 after therapy in six patients treated for ANCA-associated GN, three treated for lupus nephritis, and three treated for IgA nephropathy. All 12 patients had shown high levels of urinary FSP1 (FSP1 > 3.50 μg/g of creatinine) before therapy. However, urinary FSP1 was undetectable in 11 of those patients after successful treatment, which was judged according to the reduction of urinary protein levels and improvement of renal function. The 12th patient had lupus nephritis and continued to show proteinuria in the nephrotic range, a high titer of anti–double-stranded DNA, and severe hypocomplementemia. Moreover, urinary FSP1 levels continued to be high in this patient. This result suggests that FSP1 levels can be used as a follow-up marker.

We next measured FSP1 levels in serum samples obtained from 88 patients (14 patients with ANCA-associated GN, 38 with IgA nephropathy, 19 with minimal-change nephrotic syndrome, and 17 with membranous nephropathy) on the same day that urine samples were collected. We found that serum FSP1 levels were not elevated in the patients with ANCA-associated GN (Supplemental Figure 5), and there was no correlation between serum and urinary FSP1 levels (data not shown).

To investigate the origin of the urinary FSP1, we carried out an immunohistochemical analysis using an anti-FSP1 antibody. Figure 2A shows the three typical staining patterns. $FSP1^+$ cells accumulated

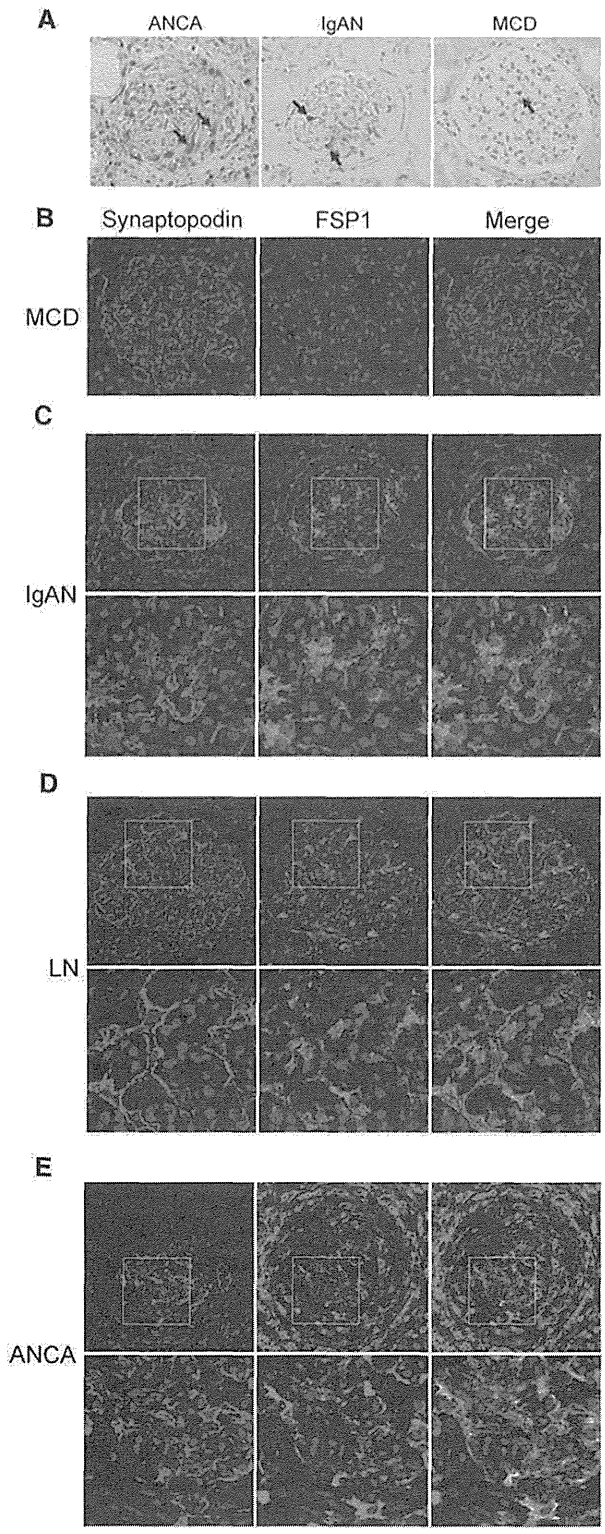

Figure 2. Increased expression of FSP1 in podocytes and cellular crescents. (A) Representative photomicrograph illustrating FSP1 expression within glomeruli. Large numbers of $FSP1^+$ cells accumulate in cellular crescents in patients with ANCA-associated glomerulonephritis (ANCA). $FSP1^+$ podocytes are localized on the outside of the glomerular basement membrane in a patient with IgA nephropathy (IgAN). $FSP1^+$ cells are rarely observed in a patient with minimal-change nephrotic syndrome (MCD). Arrows indicate $FSP1^+$ cells. (B–E) Representative immunofluorescence in renal biopsy specimens from patients with the following: (B) minimal-change nephrotic syndrome, (C) IgA nephropathy, (D) lupus nephritis (LN), and (E) ANCA. Cells expressing FSP1 (green) are clearly present within glomeruli from patients with IgA nephropathy, lupus nephritis, and ANCA. Merged images show co-localization of synaptopodin (red) and FSP1. Original magnification in upper panels, ×200. Lower panels are the high-power images of the boxed areas of the upper panels.

in cellular crescents in patients with ANCA-associated GN. $FSP1^+$ podocytes were observed in patients with IgA nephropathy. By contrast, $FSP1^+$ cell numbers in glomeruli were significantly lower in patients with minimal-change nephrotic syndrome (Figure 2, A and B). Moreover, as shown in Figure 2, C–E, dual immunofluorescence confirmed the presence of $FSP1^+$ podocytes in patients whose urinary FSP1 was higher than 1.75 μg/g of creatinine, irrespective of crescent formation. Indeed, $FSP1^+$ cell numbers and glomerular profile strongly correlated with urinary FSP1 levels (Figure 3). Taken together, these data suggest that $FSP1^+$ glomerular cells are the main source of urinary FSP1. In patients with no crescent formation, urinary FSP1 levels were significantly higher in those with more than six $FSP1^+$ podocytes than in those with fewer (Supplemental Figure 6). This finding suggests that both $FSP1^+$ podocytes and crescent-forming cells may contribute to urinary FSP1.

Identifying the origin of urinary FSP1 is difficult because elevated urinary FSP1 excretion could reflect enhanced intrarenal production, increased filtration, abnormal tubular reabsorption, or secretion from urinary cells that have detached from the renal structure. Serum FSP1 levels were not elevated in patients with crescent formation and did not correlate with urinary FSP1. Thus, urinary FSP1 excretion does not reflect increased systemic inflammation. Potential sites of FSP1 secretion into the urinary space are glomerular cells and tubular epithelial cells. The numbers of $FSP1^+$ tubular epithelial cells are much lower (<1.0/high-power field) than those of $FSP1^+$ glomerular cells, suggesting that glomerular cells are the main source of urinary

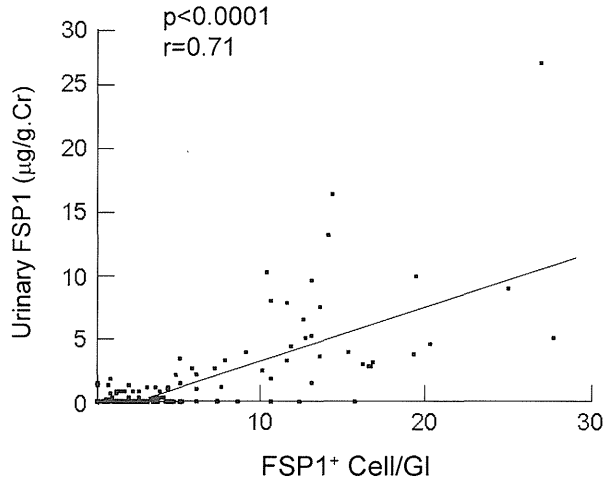

Figure 3. Urinary FSP1 levels are positively correlated with numbers of FSP1$^+$ cells/glomerular profile. Cr, creatinine; GI, glomerular profile.

FSP1. It was recently reported that FSP1 is secreted as a microparticle-like structure[15] and that podocytes are able to secrete proteins as microparticles through cellular shedding.[16] We further suggest that FSP1 secreted through microparticle shedding is not reabsorbed by tubular epithelial cells and is detected in urine samples. In addition, we previously demonstrated that more than 80% of detached podocytes express FSP1 in diabetic nephropathy, which suggests that some urinary FSP1 may be derived from detached podocytes or crescent cells. Because FSP1 was also produced by interstitial fibroblasts, the extent of renal fibrosis may have some effect on urinary FSP1 excretion. Urinary FSP1 levels weakly but significantly correlated with the extent of renal fibrosis evaluated according to the collagen type 1–positive area (Supplemental Figure 7). However, after adjustment for the number of FSP1$^+$ cells in the glomeruli, there was no association between the extent of renal fibrosis and urinary FSP1 levels.

Urinary FSP1 levels were significantly elevated in patients with high numbers of FSP1$^+$ podocytes, as well as in patients with 20% crescent formation. This finding suggests that the elevated urinary FSP1 levels are due in part to podocytes undergoing epithelial-mesenchymal transition and detaching from the glomerular basement membrane.[17–19] We previously reported that the appearance of FSP1 in podocytes of diabetic patients is associated with more severe clinical and pathologic findings of diabetic nephropathy, perhaps because of induction of podocyte detachment through an epithelial-mesenchymal transition–like phenomenon.[11] Urinary FSP1 may therefore be a novel risk factor for glomerular damage due to podocyte detachment, even in patients without crescent formation.

In summary, we established a novel ELISA system for measuring urinary FSP1, which appears to be a potentially useful biomarker for evaluating active glomerular damage, including crescent formation and the presence of FSP1$^+$ podocytes. Using this new biomarker clinically, we may be able to better identify patients who require hospitalization and urgent immunosuppressive therapy.

CONCISE METHODS

Production of Antihuman FSP1 Monoclonal Antibodies

An FSP1 expression vector was generated by cloning the full-length human *FSP1* gene into pET-49b(+) vector (Novagen, Darmstadt, Germany) carrying the GST-Tag and His-Tag sequences. BL21DE3-competent cells were then transformed using the FSP1 expression vector, after which protein expression was induced using isopropyl-β-D-thiogalactopyranoside (Takara Bio Inc., Shiga, Japan). The expressed fusion protein was purified by column chromatography using HisTrap HP columns (GE Healthcare, Tokyo, Japan), after which the GST-Tag and His-Tag were cleaved using human rhinovirus 3C protease (Novagen) to obtain the pure recombinant human FSP1 (rFSP1). The rFSP1 (50 μg/250 μl) was then emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI) and used as an immunogen.

To raise monoclonal antibodies, BALB/c mice (female, 7 weeks old) (Japan Clea, Tokyo, Japan) were intraperitoneally injected with the immunogen, and similar immunizations were carried out 2, 4, and 6 weeks later. One week after the fourth immunization, a booster injection of the immunogen without adjuvant was administered into the tail vein. Three days after the booster injection, the spleens were removed from the mice and dissociated by passage through 100-mesh steel gauze; the dissociated splenocytes (2×10^8) were then fused with an equal number of myeloma cells in the presence of 50% polyethylene glycol 1500 (Roche Applied Science, Mannheim, Germany). The fused cells were then suspended in selective growth medium supplemented with 5% Briblone (Archport, Dublin, Ireland), distributed into the wells of 96-well culture plates (2×10^5 hybrid cells/well), and cultured with periodic changes of the medium. The supernatants from wells containing hybridoma colonies were screened for the presence of specific antibodies using a direct ELISA, and the hybridoma cells from positive wells were cloned twice by limiting dilution. Each clone was then expanded in culture, after which the antibody-rich supernatants were concentrated by ammonium sulfate precipitation, dialyzed against PBS, and stored at −80°C.

Supernatants from 484 wells containing hybridoma cells were tested for antibodies. Using a direct ELISA and native PAGE, five were found to have preferential binding to rFSP1. From those we selected two clones that bound to rFSP1 with high titers (F1-2 and I11-23). Isotype analysis (Sterotec, Oxford, United Kingdom) revealed that F1-2 was of the IgG2a (κ) subclass, and I11-23 was of the IgG1 (κ) subclass. Through epitope mapping using PepSpots (JPT Peptide Technologies, Berlin, Germany), we confirmed that these two monoclonal antibodies bind to different epitopes: F1-2 recognizes the N-terminal end of

rFSP1, and I11-23 recognizes the EF hand calcium-binding domain. F1-2 was then biotinylated using NHS-LC-Biotin (Pierce Chemical, Rockford, IL), after which the efficacy of the surface biotinylation was confirmed using a 2-(4-hydroxyazobenzene) benzoic acid assay (Pierce Chemical).

Development of a Sandwich ELISA

To construct a sandwich ELISA, we coated the bottom of each well of a polyvinyl chloride microtiter plate (Thermo Labsystems, Franklin, MA) with I11-23 (1 μg/50 μl PBS) and then incubated the plate overnight at 37°C. To construct a standard curve, urine samples and rFSP1 (from 1 to 64 ng/ml) were added to each well and incubated overnight at 4°C. After the incubation, the plates were washed five times with washing buffer (PBS containing 0.05% Tween-20). The biotinylated antibody (F1-2, 1 μg/100 μl) was then added to each well and incubated for 60 minutes at 37°C. The plates were again washed five times, after which horseradish peroxidase–conjugated streptavidin (Invitrogen, Tokyo, Japan) was added to each well and incubated for 60 minutes at 37°C. After washing, o-phenylenediamine dihydrochloride (0.2 mg/ml) (Sigma-Aldrich, St. Louis, MO) with 0.02% H_2O_2 was added and incubated for 30 minutes at 37°C. The colorimetric reaction was stopped by addition of 2 M H_2SO_4 (50 μl/well), and the adsorption at 492 nm was measured with a microplate reader. Urinary concentrations were adjusted for the creatinine concentration and expressed as micrograms per gram of creatinine. The intra-assay and interassay coefficients of variation were 3.5% and 8.4%, respectively. The detection limit of the test is 1 ng/ml.

Patients and Sample Preparation

One hundred forty-seven patients (68 men and 79 women) with biopsy-proven glomerular disease were enrolled in this study after providing fully informed consent. This study was approved by our institutional review board. Patients with Henoch-Schönlein purpura nephritis, diabetes mellitus, neoplasia, viral hepatitis, amyloidosis, or other infections were excluded. The participants ranged from 16 to 89 years of age (mean age ± SD, 47.8±20.3 years). Before starting therapy, all patients were referred to the Department of Internal Medicine of Nara Medical University Hospital, and kidney biopsies were performed. Included were 19 patients with ANCA-associated GN, 10 with FSGS, 56 with IgA nephropathy, 13 with lupus nephritis, 29 with minimal-change nephrotic syndrome, and 20 with membranous nephropathy. Freshly voided urine samples were collected from each patient in the morning on the day renal biopsy was performed. Urine samples showing a urinary tract infection were excluded because of the possibility of nonspecific positivity. Macroscopic hematuria was also excluded because of the possibility of contamination by serum. Twenty-three age-matched healthy volunteers also provided urine samples. All samples were centrifuged for 10 minutes at 1500 *g* to remove any debris and were stored at −80°C before use.

Immunohistochemistry

Renal biopsy specimens were fixed in 10% buffered formalin for 12 hours, dehydrated, embedded in paraffin, and sectioned according to standard procedures. The sections were then deparaffinized and incubated with proteinase K (0.4 mg/ml) for 5 minutes at room temperature for FSP1 staining or were incubated with 0.1% trypsin for 90 minutes at 37°C for collagen type 1 staining. The endogenous peroxidase activity was then blocked with 0.03% hydrogen peroxide, and nonspecific protein binding was blocked with 5% normal goat serum in PBS containing 2% BSA. The blocked sections were incubated for 60 minutes at room temperature with a primary rabbit polyclonal antihuman FSP1 antibody (1:5000 dilution) or with a primary rabbit polyclonal antihuman collagen type 1 antibody (1:500 dilution; Abcam, Cambridge, MA), after which the antibody was detected using a DAKO Envision+System peroxidase (diaminobenzidine) kit (DakoCytomation Inc., Carpinteria, CA). The sections were then counterstained with hematoxylin. The specificity of FSP1 staining was confirmed using control rabbit serum and by absorption of the anti-FSP1 antibody using an excess of rFSP1 protein. The area positively stained for collagen type 1 was calculated using AnalySIS image analysis software (Soft Imaging System, Munster, Germany).

Frozen sections of renal biopsy specimens were also stained for dual immunofluorescence microscopy. After the sections were fixed on glass slides in 4% paraformaldehyde for 15 minutes at 4°C, they were incubated for 60 minutes, first with goat polyclonal antihuman synaptopodin (P-19) antibody (1:500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then with rabbit polyclonal antihuman FSP1 antibody (1:2000 dilution).[13] The sections were then washed three times with PBS and incubated for 30 minutes with DyLight 488-conjugated donkey anti-rabbit secondary antibody (1:800 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and a Cy3-conjugated donkey antigoat secondary antibody (1:1000 dilution; Jackson Immuno Research Laboratories Inc.). Finally, the sections were counterstained with 4′6-diamidine-2′- phenylindole dihydrochloride (Molecular Probes Inc., Eugene, OR) and viewed under a confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan).

Statistical Analyses

Data were recorded as the median (25th percentile, 75th percentile), and $P<0.05$ was considered to represent a statistically significant difference. The Mann–Whitney *U* test was used for comparisons between two groups. The Kruskal–Wallis test with *post hoc* analysis using the Mann–Whitney test and adjustment of the *P* value using the Bonferroni method ($P<0.002$) was used to assess differences in clinical measures among more than three groups. Pearson correlation coefficients were used to assess relationships between urinary FSP1 and $FPS1^+$ cell number and glomerular profile. All analyses were performed using JMP 5.1 software (SAS Institute, Cary, NC). Sensitivity, specificity, and predictive values were calculated using receiver-operator characteristic curves and 2×2 tables.

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DISCLOSURES

None.

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Nutrient Sensing, Autophagy, and Diabetic Nephropathy

Shinji Kume,[1] Merlin C. Thomas,[2] and Daisuke Koya[3]

The prevalence of diabetic nephropathy, a serious complication of diabetes, has been increasing worldwide. Therefore, there is an urgent need to identify a new therapeutic target to prevent diabetic nephropathy. "Nutrient-sensing" pathways are generally well conserved among eukaryotes. Accumulating evidence indicates that alteration of nutrient-sensing pathways and subsequent impairment of cell function in insulin-sensitive organs of mammals are involved in the pathogenesis of type 2 diabetes. According to recent reports, nutrient-sensing in the kidney also seems to be altered under diabetic conditions. In this review, we discuss the possibility that nutrient-sensing pathways can be a therapeutic target for diabetic nephropathy and suggest future directions for research.

NUTRIENT SENSING, AUTOPHAGY, AND DIABETIC NEPHROPATHY

Each cell has the ability to recognize and specifically respond to nutrient fuel substrates, such as glucose, lipids, and amino acids, to ensure their efficient use. These nutrient-sensing pathways appear critical for cellular homeostasis, for coping with starvation, and making the most of nutrient abundance. These pathways also represent important regulators of cell growth and proliferation, motility, mitochondrial function, autophagy, and survival (1–4).

Nutrient sensing is highly conserved across eukaryotic species. These pervasive regulatory pathways use posttranslational modifications of target proteins to link substrate availability to cellular homeostasis and stress responses (1–4). The best known of these pathways include the mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and the sirtuins (SIRT). Under low-energy conditions, AMPK and SIRT are activated by increases in intracellular AMP and NAD^+ levels, respectively (1,2). In excessive nutrient conditions, mTOR is activated (3,4).

Each of these nutrient-sensing pathways has been implicated in the pathogenesis of obesity and diabetes, including actions on β-cell, adipocyte, hepatic and skeletal muscle metabolism, and the central regulation of nutrition (1–4). However, the same pathways may also be directly relevant to the development and progression of diabetes complications (Fig. 1). In particular, the cells of the kidney appear especially vulnerable to the effects of over-nutrition, conveyed via the aforementioned energy-sensing pathways (5–7).

Autophagy, a lysosomal degradation pathway, plays a crucial role in removing protein aggregates as well as damaged organelles to maintain intracellular homeostasis during various stress conditions (8,9) that are involved in the pathogenesis of diabetic nephropathy. Interestingly, autophagy is regulated by the above-mentioned nutrient-sensing pathways (9–11). Thus, alteration of these nutrient-sensing pathways under diabetic conditions may impair the autophagic response stimulated by intracellular stress, which may lead to exacerbation of organelle dysfunction and subsequently result in diabetic nephropathy.

These findings led us to hypothesize that these signaling pathways are involved in pathogenesis of diabetic nephropathy and may be a potential therapeutic target for the prevention of diabetic nephropathy. Here, we provide a review and perspective regarding the potential actions of nutrient-sensing pathways and autophagy in diabetic nephropathy.

mTOR IN DIABETIC NEPHROPATHY

Of the many nutrient-sensing pathways, mTOR has received the most attention for its potential actions in diabetic nephropathy. mTOR (also known as FK506 binding protein 12-rapamycin associated protein 1 or FRAP1) is a serine/threonine protein kinase whose activity is associated with cellular nutrient levels and redox status (12,13). An excess of nutrient components increases the activity of mTOR, either by direct interactions (i.e., in response to glucose or amino acids) or indirectly via metabolic signaling pathways, including insulin and other growth factors (4,12,13).

mTOR provides the enzymatic activity for two distinct protein complexes. The best characterized is mTOR complex 1 (mTORC1), which is composed of mTOR, regulatory-associated protein of mTOR (raptor), mammalian LST8/G-protein β-subunit–like protein (mLST8/GβL), PRAS40, and DEP domain protein that interacts with mTOR (DEPTOR) (4,12). mTORC1 receives nutrient input via small GTPases, such as Rag and Rheb (14,15). An increase in amino acid levels and growth factors activate mTORC1 via Rag and Rheb activation, respectively. Activation of mTORC1 results in phosphorylation of ribosomal S6 kinase (S6K) and eukaryotic translation-initiation factor 4E-binding protein on several sites (4,12). This stimulates ribosome biogenesis and protein synthesis, subsequently leading to cell growth. mTORC1 activity is inhibited by the bacterial toxin rapamycin (marketed as sirolimus). Indeed, the anticell growth effect of sirolimus is thought to be largely mediated by its ability to prevent translation initiation.

Diabetic nephropathy is characterized by dysregulated cell growth and cell-cycle participation, with uncontrolled hypertrophy, exaggerated proliferative responses, and increased apoptosis in mesangial and proximal tubular cells (16). In the diabetic kidney, there is significant early activation of mTOR-dependent pathways in both animal models and humans with diabetes (17–24). Moreover, podocyte-specific

From the [1]Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan; the [2]Baker IDI Heart and Diabetes Institute, Melbourne, Australia; and the [3]Division of Diabetes and Endocrinology, Kanazawa Medical University, Kahoku-Gun, Ishikawa, Japan.
Corresponding author: Daisuke Koya, koya0516@kanazawa-med.ac.jp.
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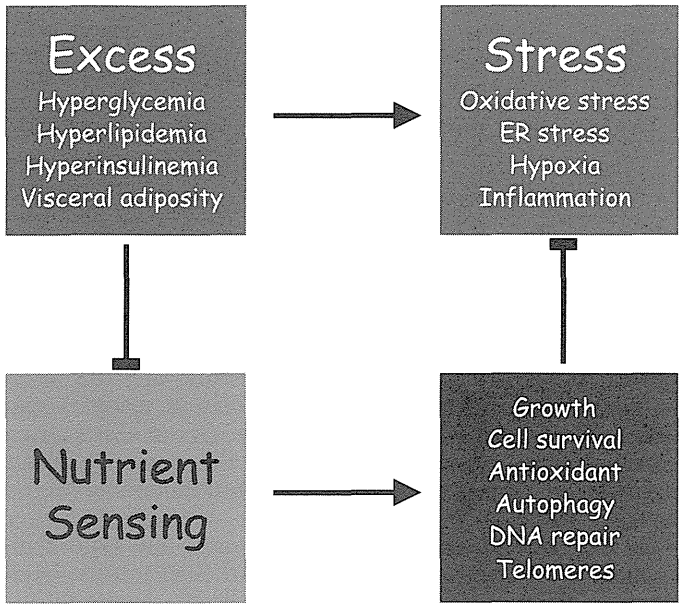

FIG. 1. The potential role of nutrient-sensing pathways in the pathogenesis of diabetes complications.

activation mTORC1 recapitulates many features of diabetic nephropathy, including mesangial expansion and proteinuria (25). The mTOR inhibitor rapamycin has also been extensively studied in experimental diabetic nephropathy. Beneficial actions on tubular and glomerular hypertrophy, mesangial expansion, glomerular basement-membrane thickening, tubular epithelial-to-mesenchymal transition, macrophage recruitment, and reduction in proteinuria have been reported (17–24) (Table 1). To what extent these findings reflect its direct renal actions, or indirect effects to the systemic immune system or glucose-lipid metabolism, continues to be debated. However, recent studies using podocyte-specific repression of raptor, a component of mTORC1, to attenuate mTOR signaling have demonstrated benefits with respect to proteinuria, glomerulosclerosis, and mesangial matrix expansion in murine models of diabetes (25,26).

Other studies have reported an increase in proteinuria and glomerulosclerosis following treatment with the mTOR inhibitor rapamycin in some patients and selected animal models (27,28). Certainly, mTORC1 activity modulates the expression of podocyte slit diaphragm proteins and cytoskeleton structure in vitro (29). In addition, outside diabetes, podocyte-specific deletion of mTORC1 signaling also results in progressive glomerulosclerosis (25,26), with a phenotype similar to that observed in podocyte-specific insulin receptor–deficient mice (it should be remembered that insulin stimulates mTOR signaling) (30). Taken together, these data suggest that achieving a level of mTOR signaling consistent with energy and cellular stress may be the best way to achieve renal health, and that functionally too much or little mTOR may have its own physiological consequences.

In addition, mTOR is the catalytic unit for a second protein complex, the mTOR complex 2 (mTORC2), which also contains the rapamycin-insensitive companion of mTOR (rictor), mLST8/GβL, mammalian stress-activated protein kinase interacting protein 1 (mSIN1), and Protor-1 or Protor-2 (31). Although, mTORC2 is also activated by growth factors, unlike the mTORC1, its actions are rapamycin insensitive, at least in the short term (31). Consequently, the overall effects of mTOR in the diabetic kidney cannot simply be inferred from studies with rapamycin. The exact role and regulatory mechanisms of mTORC2 are less well understood, and few studies have investigated the role of mTORC2 in the kidney. Unlike mTORC1 knockout, mTORC2 knockout, specifically in the podocytes of nondiabetic mice, shows no phenotype, which would suggest that the mTORC2 function in podocytes is not essential (26). However, mTORC2 in podocytes plays a protective role against stress-induced damage to the filtration barrier (26). Consistent with this finding, the phenotypes in double

TABLE 1
Studies demonstrating the potential renal actions of mTORC1-dependent signaling in experimental diabetic nephropathy

Experimental type	Renal outcome/phenotype	Mechanism	Reference
S6 kinase $1^{-/-}$ mice	Renal hypertrophy↓	Inhibition of p70S6 kinase	Chen et al. (17)
Rapamycin (*db/db* mice)	Renal and glomerular hypertrophy↓	eEF2 kinase phosphorylation and laminin β1 expression	Sataranatarajan et al. (22)
Rapamycin (STZ-diabetic mice)	Renal hypertrophy↓	Inhibition of p70S6 kinase	Sakaguchi et al. (21)
Sirolimus (STZ-diabetic rats)	Glomerular hypertrophy↓, podocyte loss↓	Decreases of TGF-β and VEGF expression	Wittmann et al. (23)
Rapamycin (*db/db* mice)	Albuminuria↓, glomerular lesion↓	Inhibition of p70S6 kinase	Mori et al. (19)
Rapamycin (STZ-diabetic rats)	Albuminuria↓, glomerular lesion↓, inflammation↓	Decreases of TGF-β, VEGF and MCP-1 expression	Yang et al. (24)
Rapamycin (STZ-diabetic rats)	Albuminuria↓, glomerular lesion↓	Decreases of TGF-β, CTGF and α-SMA expression	Lloberas et al. (18)
$Gas6^{-/-}$ mice	Glomerular hypertrophy↓, mesangial expansion↓	Inhibition of Akt and p70S6 kinase	Nagai et al. (20)
Podocyte-specific TSC1 knockout mice	Albuminuria↑, glomerular lesion↑	Activation of mTORC1	Inoki et al. (25)
Podocyte-specific raptor knockout mice	Albuminuria↓, glomerular lesion↓	Inactivation of mTORC1	Gödel et al. (26)

CTGF, connective tissue growth factor; MCP-1, monocyte chemo-attractant protein-1; PARP, poly(ADP-ribose) polymerases; PTECs, proximal tubular epithelial cells; α-SMA, α-smooth muscle actin; STZ; streptozotocin; TGF-β, transforming growth factor β; TSC, tuberous sclerosis complex; VEGF, vascular endothelial growth factor.

knockout of mTORCs in podocyte are more severe than in single mTORC1 knockout; this supports the notion that mTORC2 exerts its protective role against podocyte injury induced by mTORC1 depletion (26). mTORC2 also phosphorylates serum- and glucocorticoid-induced protein kinase 1, the serine/threonine protein kinase Akt/protein kinase B, and protein kinase C (PKC) (31), all of which are known to be crucial causal factors for the development and progression of diabetic nephropathy; this suggests that mTORC2 may be involved in the pathogenesis of diabetic nephropathy. However, further investigations need to be conducted to clarify this issue.

In the diabetic kidney, the pathogenic role of mTOR in the proximal tubules is still poorly understood. Cellular hypertrophy and apoptosis in the proximal tubules are the main characteristics of diabetic nephropathy (21,32). High glucose-induced mTOR activation is associated with both phenotypes in the proximal tubular cells (21,32). mTOR could be involved in the pathogenesis of tubular lesions in diabetic nephropathy, although further studies using proximal tubule-specific mTOR knockout mice are necessary to confirm this.

SIRT1 IN DIABETIC KIDNEY DISEASE

Sirtuins (silent information regulator 2 [Sir2]) are protein deacetylases that respond to changing cellular $NADI^+$ levels associated with metabolic and redox stresses by deacetylating proteins that contribute to cellular regulation, adaptation, and survival (1). Sirtuins are best known for their acknowledged link with longevity associated with calorie restriction as well as being the putative target of the plant-derived polyphenol resveratrol (1). There are seven mammalian sirtuins (SIRT1 to SIRT7), which share a conserved NAD^+ binding and catalytic core domain, but which differ in acylprotein substrate specificity, binding partners, and intracellular localization (1). The most studied mammalian sirtuin is SIRT1, which has been broadly implicated in a range of metabolic processes, including lipid and glucose metabolism and weight regulation/adiposity (1). Under conditions of calorie restriction, increased intracellular NAD^+ levels promote the activity of SIRT1, leading to changes in energy metabolism and the stimulation of stress-resistance pathways, including antioxidant release, DNA repair, telomere maintenance, and autophagy (1,11). The overexpression of SIRT1 in transgenic mice results in lower body weight, greater metabolic activity, and reduced glucose and lipid levels—phenotypic features similar to those observed in calorie-restricted mice (33). By contrast, high-fat diet–induced insulin resistance and hyperglycemia are all associated with decreased expression and activity of SIRT1 (1).

Although most research emphasis has been placed on the actions of SIRT1 in cancer, metabolism, and aging, the dysregulation of growth and stress responsiveness associated with diabetic kidney disease are also potentially linked to SIRT1 expression. In the kidney, SIRT1 is preferentially expressed in the inner medulla and renal interstitium, where it is thought to protect against oxidative stress, partly through induction of cyclooxygenase-2 (34). SIRT1 deficiency accentuates renal fibrosis following unilateral ureteral obstruction (UUO), whereas treatment with SIRT1 activators decreases renal apoptosis and fibrosis after UUO injury (34). Consistent with these "antioxidant" actions, overexpression of SIRT1 in proximal tubular epithelial cells increases the expression of the intracellular antioxidant catalase, which confers protection against cisplatin-induced injury (35). Antiapoptotic effects of SIRT1 overexpression have also been reported in cultured mesangial cells (36,37).

Like the UUO model, diabetic nephropathy is characterized by progressive and cumulative atrophy and apoptosis of tubular epithelial cells, which precedes manifestations including tubular dilatation, peritubular fibrosis, and subsequent nephron dropout. In the diabetic kidney, up to 51% of glomeruli may be attached to atrophic tubules, and up to 17% of glomeruli may be "atubular" (38). Although a number of metabolic and hemodynamic changes associated with diabetes can modulate the expression of apoptosis-regulatory genes, among the most important effectors appears to be oxidative stress, since tubular apoptosis in diabetes can be partly prevented by antioxidants. Given the known tubuloprotective effects of SIRT1 in other models, recent researchers have also explored its potential actions in the diabetic kidney.

In experimental models of both type 1 and type 2 diabetes, the expression and activity of SIRT1 have been reported to be reduced in the kidney (39,40). Nonetheless, studies using SIRT1 activators in models of both type 1 and type 2 diabetes have reported renoprotective benefits (41,42). For example, we have recently shown that resveratrol treatment in *db/db* mice, a model of type 2 diabetes, results in improved renal functional and histological abnormalities, such as albuminuria, mesangial expansion, glomerular and interstitial fibronectin accumulation, and interstitial macrophage infiltration (42). However, in this study, resveratrol also reduced intracellular reactive oxygen species in SIRT1–knocked-down cells, suggesting that its actions to exert antioxidative effects, at least in proximal cells, were independent of the SIRT1 signaling (42). Indeed, recent studies have suggested that resveratrol and the other small molecules (SRT1720, SRT2183, SRT1460) may not be direct activators of SIRT1, but mediate their physiological effects via off-target activities (43). Consequently, the true role of SRT in the diabetic kidney or potential benefits from increasing SIRT1 activity remains to be established. Nonetheless, overexpression of SIRT1 in transgenic mice is able to reduce circulating levels of proinflammatory cytokines, adipokines, and other prooxidant molecules associated with chronic exposure to a high-fat diet (44). Insofar that these mediators also contribute to renal damage in type 2 diabetes, it is likely that at the very least the development of legitimate SIRT1 agonist may prove beneficial in the diabetic kidney, albeit indirectly.

There is also emerging evidence that SIRT1 participates in the regulation of sodium balance and blood pressure control. Diabetes is associated with an increase in fractional and absolute sodium reabsorption in the proximal tubule. This is partly determined by renal hypertrophy, which increases proximal salt reabsorption simply by mass action. Indeed, inhibitors of tubular growth reduce salt reabsorption and hyperfiltration in direct proportion to their effect on kidney size (45). However, an important additional stimulus for salt reabsorption may be excess energy levels, mediated via nutrient sensors such as SIRT1. That there is a fundamental link between active (energy intensive) reabsorption of salt and energy supplies is hardly surprising, given that the oxygen consumption/tissue weight by the kidney is exceeded only by that of the beating heart. Salt reabsorption is significantly down-regulated following calorie restriction, partly mediating its antihypertensive

effect. SIRT1 also represses the expression of apical sodium channel, ENaC (46). For the same reasons, it is possible to speculate that energy excess associated with diabetes and obesity may contribute to avid sodium retention, hyperfiltration, and hypertension.

AMPK IN DIABETIC KIDNEY DISEASE

AMPK is a ubiquitously expressed heterotrimeric kinase that plays a key role in cellular energy homeostasis (2). AMPK consists of an α, a β, and a γ subunit and is activated by the upstream kinases such as calcium calmodulin-dependent protein kinase kinase (CaMKK) and serine/threonine kinase 11 (LKB1) via phosphorylation of threonine residue 172 (47). Activation of AMPK by CaMKK and LKB1 is dependent, respectively, on intracellular calcium and the AMP:ATP ratio (47). During energy-depleted conditions, intracellular concentrations of AMP rise while ATP levels fall, leading to increased activation of AMPK and phosphorylation of its multiple substrates to enhance catabolism and suppress anabolic energy consumption. In excess energy states, reduced AMPK activation stimulates protein synthesis, cell growth, and storage. AMPK activity is also independently regulated by circulating hormones and cytokines, including bradykinin and the adipokines leptin and adiponectin (2,47). AMPK is also induced by the antidiabetic agent metformin, thiazolidinediones, and the peroxisome proliferator–activated receptor α agonist fenofibrate, which may partly contribute to these renoprotective actions in diabetic nephropathy (2,47–50).

The metabolic effects of AMPK activation have been extensively characterized and include effects on glucose and lipid metabolism, mitochondrial function, and exercise-induced glucose utilization. However, the actions of AMPK in the kidney are less well understood. AMPK is widely expressed in all renal cell types, including podocytes, mesangial cells, glomerular endothelial cells, and tubular cells, especially the mitochondrial rich cells of the proximal tubule and thick ascending limb of the loop of Henle. The activity of AMPK in the kidney appears to be reduced in experimental models of diabetes (42).

One of the earliest structural changes in the diabetic kidney is hypertrophy and hyperplasia of the tubuli of the cortex and the outer medulla. Early studies have demonstrated that diabetic rats exhibit a 15% increase in whole kidney weight within 72 h of induction of diabetes with streptozotocin (51). Most of this increase may be accounted for by proliferation, hypertrophy, and elongation of proximal tubular cells. These changes appear to be driven by tubular glucose reabsorption because they are attenuated by phlorizin, an inhibitor of Na^+/glucose cotransport (52). However, the mechanism(s) by which increased tubular glucose flux is sensed and subsequently stimulates the increased production of growth factors and suppresses antiproliferative mediators is a matter of ongoing research. One possible candidate for the role of "metabolic master-switch" is AMPK (5). Exposure of tubular cells to hyperglycemia in vitro results in reductions in AMPK phosphorylation and cellular hypertrophy that can be inhibited by metformin and AICAR, while expression of kinase-inactive AMPK augments glucose-induced protein synthesis (53). Similarly, in experimental models of type 1 diabetes, pharmacological activation of AMPK has been shown to attenuate renal hypertrophy (53). However, the renal actions of metformin, like those of resveratrol, are probably more complicated, with a range of effects that appear to be independent of AMPK, since the effects are also observed in AMPK-deficient tubular cells and not reproduced by the AMPK agonist, AICAR (53).

The accumulation of intracellular glycogen granules (the so-called Armanni-Ebstein lesion) is perhaps the best-known tubular change associated with diabetes. Its pathological significance remains to be established, although it is widely considered to be injurious. Certainly, the Fanconi-Bickel syndrome, a disorder associated with tubular glycogen accumulation due to a mutation of the GLUT-2 transporter, is also associated with progressive diabetic-like changes in the glomeruli (54). Large glycogen accumulations are thought to alter the cellular architecture with loss of basal infoldings and apical microvilli and, ultimately, caspase-mediated apoptosis. One of the metabolic signals stimulating tubular glycogen synthesis in the diabetic kidney appears to be reduced tubular AMPK activity since AMPK directly phosphorylates and inactivates glycogen synthase (55). AMPK deficiency may also contribute to triglyceride accumulation through suppressed lipolysis and enhanced renal lipogenesis, in part mediated by reduced AMPK-mediated phosphorylation of the lipogenic enzyme acetyl-CoA carboxylase (2,47).

AMPK may also partly mediate the renal effects of the abundant circulating adipokine adiponectin. Serum adiponectin concentrations are increased in individuals with type 1 diabetes, especially those with chronic kidney disease (56). Moreover, elevated adiponectin levels are associated with increased risk of progressive nephropathy in adults with type 1 diabetes (56). It has been suggested that this elevation may be a response to renal injury, rather than its cause. Certainly, adiponectin is able to activate AMPK in podocytes, mesangial cells, and glomerular endothelial cells, which leads to cytoprotective responses, including reduced oxidative stress (57). Furthermore, adiponectin knockout mice exhibit increased albuminuria and effacement of podocyte foot processes (58). Similarly, administration of adiponectin to cultured podocytes leads to increased AMPK activity and reduced permeability to albumin. By contrast to type 1 diabetes, adiponectin levels are reduced in patients with type 2 diabetes, reflecting its link with visceral adiposity. Because diabetic nephropathy may therefore occur with both low and high adiponectin levels, any direct role for adiponectin in diabetic kidney disease appears less likely.

Finally, AMPK is also implicated in the regulation of tubular sodium reabsorption, the most energy-intensive process in the kidney (5). In order to ensure efficient tubular functions, it is thought that AMPK maintains tight coupling between energy metabolism and tubular transport. AMPK activation in low-energy states leads to the inactivation of key ion transporters, including the cystic fibrosis transmembrane conductance regulator, the epithelial sodium channel, the Na^+-K^+-$2Cl^-$ cotransporter, and the vacuolar H^+-ATPase (5). By contrast, reduced AMPK activity, as is observed in diabetes, is associated with increased tubular sodium reabsorption. This may be important for the pathogenesis of hyperfiltration and, ultimately, salt-dependent hypertension.

AUTOPHAGY IN DIABETIC RENAL DISEASE

Autophagy is the catabolic process by which intracellular components are degraded through the lysosomal machinery (8,9). Autophagy plays a critical role in removing components that have become damaged or dysfunctional as a result

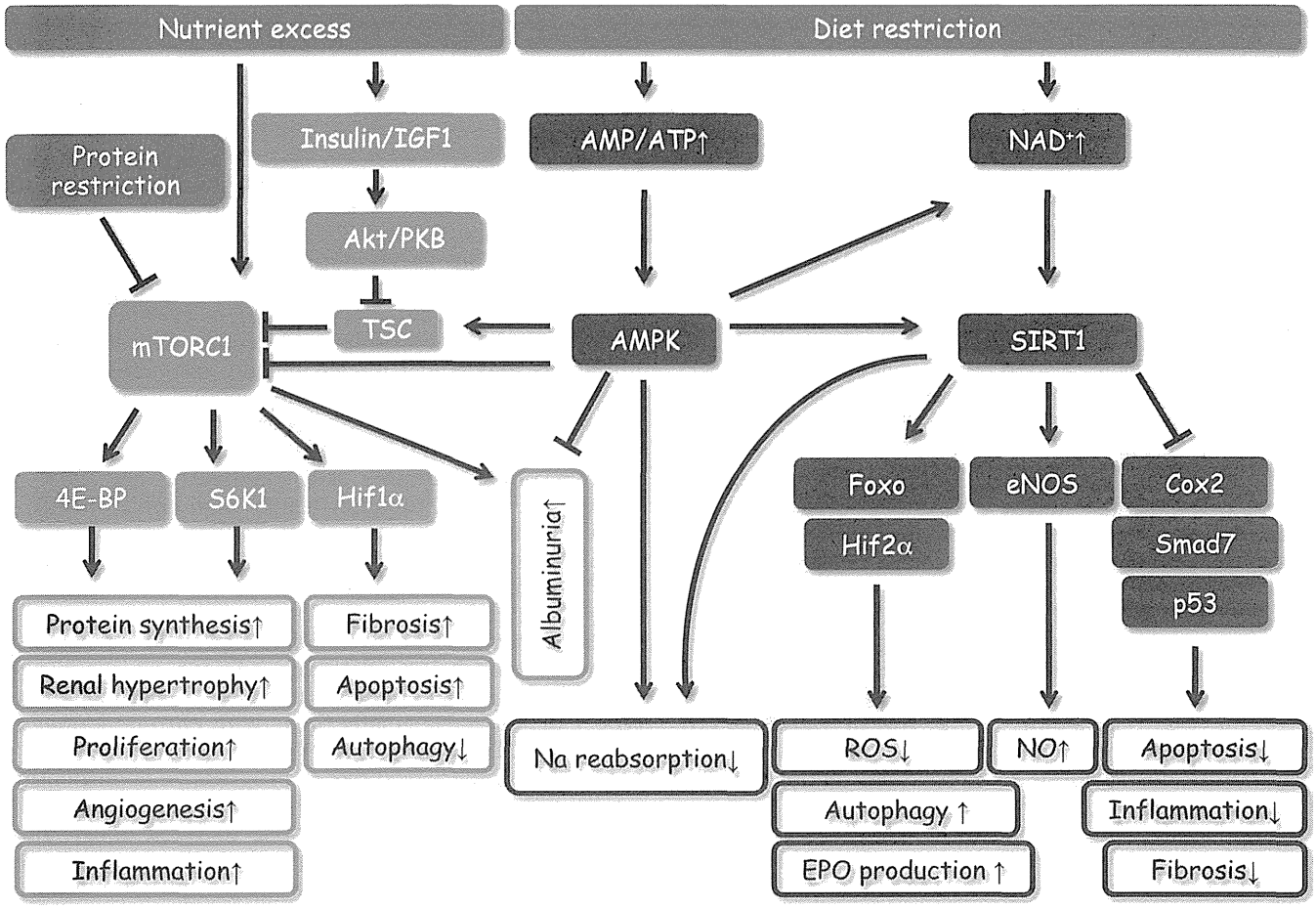

FIG. 2. The three nutrient-sensing pathways, mTOR, AMPK, and SIRT1, may be independently and coordinately involved in the pathogenesis of diabetic nephropathy. 4E-BP, 4E-binding protein; COX2, cyclooxygenase-2; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; FoxO, forkhead box class O; Hif2α, hypoxia-inducible factor 2α; IGF1, insulin-like growth factor 1; NO, nitric oxide; PKB, protein kinase B; ROS, reactive oxygen species; TSC, tuberous sclerosis complex.

of exposure to cellular stressors (8,9). However, its actions in health and disease are far more complicated than simply replacement and renewal. Autophagy is tightly regulated to ensure an optimal balance between the synthesis and degradation, use, and storage and recycling of cellular products. Even healthy components may be jettisoned for the greater good of cellular homeostasis. One of the chief regulators of autophagy is cellular energy levels that determine which components are essential via the above-mentioned nutrient-sensing pathways (7,9–11). If energy levels become depleted, autophagy is activated to pare down energy expenditure and provide substrate resources for cells. For example, inhibition of mTOR or activation of AMPK and SIRT1, which occurs during nutrient starvation, activates nonselective autophagy (10,11).

When renal cells are exposed to conditions that lead to stress and injury, including hypoxic, genotoxic, oxidative, and endoplasmic reticulum (ER) stress, autophagy is upregulated to maintain cellular homeostasis via the degradation/turnover of cytoplasmic components, such as damaged proteins and organelles (3,9). For example, ER dysfunction results in the generation of misfolded proteins, which accumulate if their rate of production exceeds the readiness and capacity for autophagy. Equally, exposure of podocytes to angiotensin II increases autophagy through increased oxidative stress (59). In these settings, autophagy is an important survival factor, without which stress-induced apoptosis would be more likely. Autophagy is especially important for maintenance of postmitotic cells, such as podocytes, which have only limited capacity for regeneration. Indeed, podocyte-specific deletion of autophagy-related 5 leads to proteinuria and glomerulopathy in aging mice (60).

In states of energy excess, autophagy is downregulated to make the most of nutrient abundance. Although in the short term this is beneficial, the failure of autophagy may ultimately contribute to the accumulation of cell damage and aging (7,60). Autophagy deficiencies may also contribute to increased chronic renal injury associated with hypoxia (7), ischemia-reperfusion (61), and cisplatin-induced damage (62), partly by promoting apoptosis. In the hypertrophic diabetic kidney, not only is the capacity for autophagy reduced, but because there is high exposure to cellular stresses, the need for cytoprotective autophagy is significantly increased. Diabetic kidney disease is associated with the intracellular accumulation of periodic acid-Schiff–positive lysosomal dense bodies, chiefly in the straight proximal tubules (S2 and S3). These lysosomal bodies, containing multilamellar inclusions, are classically considered to be accumulated phospholipid membranes indicative of membrane damage and lipid peroxidation. However, more than simply as markers of injury, some of these granules may be independently involved in the

generation of intracellular oxidative stress and cellular dysfunction (63).

Although autophagy has beneficial actions, it is also suggested to be one means of programmed cell death (known as type II cell death). Whether autophagy is causally related to cell death or represents a cell's ultimate effort for survival is unclear. Certainly when apoptosis is inhibited, autophagic cell death can be induced, possibly via oxidative stress. The role or existence of autophagic cell death in the diabetic kidney has not been established.

DIABETES: A RENAL PERSPECTIVE

From a kidney's point of view, diabetes is a bonanza state of nutrient excess. Even when glucose and lipids are under control, signals from the brain, fat, liver, and other metabolic sites remind the kidney of the surfeit and the need to "make hay while the sun shines." This occurs despite increased cellular stresses, which should promote autophagy, regeneration, and biogenesis and would normally serve to slow down metabolism. It is possible to speculate that these mixed messages, and ultimately their disconnection, contribute to renal dysfunction in diabetes (Fig. 1).

How can we protect the kidney against diabetes? Multitarget modulation of nutrient sensors may be one possible target to enhance autophagy and promote the tissue repair required to attenuate renal damage in diabetes (Fig. 2). As a potential proof of concept, intermittent fasting in a streptozotocin-induced and Wister fatty rats is associated with increased renal SIRT1 activity and renoprotection (40,64). Although such nutritional interventions in clinical diabetes are impractical, multitarget interventions directed against nutrient-sensing pathways to simulate starvation represent an emerging new therapeutic approach for the prevention and management of diabetic nephropathy. However, these pathways play a critical role in cellular differentiation as well as anabolic processes that are required for the maintenance of functional organisms. Therefore, further investigations, especially ones focusing on adequate levels and tissue/cell-specificity of manipulation of these pathways, are needed.

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Review Article

Autophagy as a Therapeutic Target in Diabetic Nephropathy

Yuki Tanaka,[1] Shinji Kume,[1] Munehiro Kitada,[2] Keizo Kanasaki,[2] Takashi Uzu,[1] Hiroshi Maegawa,[1] and Daisuke Koya[2]

[1] *Department of Medicine, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan*
[2] *Division of Diabetes & Endocrinology, Kanazawa Medical University, Kahoku-Gun, Ishikawa 920-0293, Japan*

Correspondence should be addressed to Daisuke Koya, koya0516@kanazawa-med.ac.jp

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Diabetic nephropathy is a serious complication of diabetes mellitus, and its prevalence has been increasing worldwide. Therefore, there is an urgent need to identify a new therapeutic target to prevent diabetic nephropathy. Autophagy is a major catabolic pathway involved in degrading and recycling macromolecules and damaged organelles to maintain intracellular homeostasis. The study of autophagy in mammalian systems is advancing rapidly and has revealed that it is involved in the pathogenesis of various metabolic or age-related diseases. The functional role of autophagy in the kidneys is also currently under intense investigation although, until recently, evidence showing the involvement of autophagy in the pathogenesis of diabetic nephropathy has been limited. We provide a systematic review of autophagy and discuss the therapeutic potential of autophagy in diabetic nephropathy to help future investigations in this field.

1. Introduction

The prevalence of diabetes mellitus has been increasing worldwide during recent years, and this is estimated to continue in the future [1, 2]. Diabetic nephropathy is a serious complication of diabetes mellitus and is the most common cause of end-stage renal disease [3, 4]. The increasing prevalence of diabetes mellitus and its complications, including diabetic nephropathy, has therefore become a major health problem worldwide. There is now an urgent need to identify new therapeutic target molecules or cellular processes that underlie the pathogenesis of diabetic nephropathy to establish an additional therapeutic option.

Hyperglycemia-mediated alteration of extra- and intracellular metabolism, such as advanced glycation end products [5], increased protein kinase C activity [6], and abnormal polyol metabolism [7], has been recognized as classical pathogenesis of diabetic nephropathy. In addition, intracellular stress associated with renal hypoxia [8, 9], mitochondrial reactive oxygen species (ROS) [10–13], and endoplasmic reticulum (ER) stress [14–16] has recently been proposed and focused as new pathogenesis of diabetic nephropathy. Thus, to maintain the cellular homeostasis against stress condition derived from organelle dysfunction or hypoxia may be a new therapeutic target of diabetic nephropathy.

Autophagy, a lysosomal protein degradation pathway in cells, plays a crucial role in removing protein aggregates as well as damaged or excess organelles to maintain intracellular homeostasis and cell integrity [17]. It has recently been highlighted because it can be stimulated by multiple types of cellular stressors including starvation, hypoxia, or ER stress. The study of autophagy in mammalian systems and in disease states is advancing rapidly, and many investigators are entering this new and exciting field (Figure 1). It has been revealed that autophagy plays a crucial role in several organs, especially in metabolic organs, and that its alteration is involved in the pathogenesis of metabolic [18–21] and age-related diseases [22–27]. The functional role of autophagy in the kidneys is currently under intense investigation (Figure 1), and it has been revealed that autophagy has a renoprotective role in several animal models including those used for aging and acute kidney injury [26–31]. However, the role of autophagy in diabetic nephropathy remains unclear.

Alteration of several nutrient-sensing pathways is related to the development of metabolic diseases, such as type 2 diabetes and its vascular complications. Major nutrient-sensing