The effect of dexamethasone on defective nephrin transport caused by ER stress: A potential mechanism for the therapeutic action of glucocorticoids in the acquired glomerular diseases

Y Fujii[1], J Khoshnoodi[2], H Takenaka[3], M Hosoyamada[4], A Nakajo[5], F Bessho[5], A Kudo[6], S Takahashi[7], Y Arimura[1], A Yamada[1], T Nagasawa[1], V Ruotsalainen[8], K Tryggvason[9], AS Lee[10] and K Yan[5]

[1]Department of Internal Medicine, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [2]Department of Medicine, Division of Nephrology and hypertension, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; [3]Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [4]Department of Toxicology and Pharmacology, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [5]Department of Pediatrics, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [6]Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [7]Department of Pediatrics, Nihon University, Chiyoda-ku, Tokyo, Japan; [8]Biocenter Oulu and Department of Biochemistry, University of Oulu, Oulu, Finland; [9]Department of Medical Biochemistry and Biophysics, Division of Matrix Biology, Karolinska Institute, Stockholm, Sweden and [10]Department of Biochemistry and the Norris Cancer Research Institutes, University of Southern California School of Medicine, Los Angeles, California, USA

The mechanism by which glucocorticoids govern antiproteinuric effect in nephrotic syndrome remains unknown. Present study examined the protective role of dexamethasone (DEX) in the intracellular trafficking of nephrin under endoplasmic reticulum (ER) stress. Human embryonic kidney-293 cell line expressing a full-length human nephrin was cultured in mediums containing 5.5 or 25 mM glucose with or without DEX. The result revealed that glucose starvation evoked a rapid ER stress leading to formation of underglycosylated nephrin that was remained in the ER as a complex with calreticulin/calnexin. DEX rescued this interfered trafficking through binding to its receptor and stimulating the mitochondrial transcripts and adenosine 5′ triphosphate (ATP) production, leading to synthesis of fully glycosylated nephrin. These results suggest that ER-stress in podocytes may cause alteration of nephrin *N*-glycosylation, which may be an underlying factor in the pathomechanism of the proteinuria in nephrotic syndrome. DEX may restore this imbalance by stimulating expression of mitochondrial genes, resulted in the production of ATP that is essential factor for proper folding machinery aided by the ER chaperones.

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Correspondence: *K Yan, Department of Pediatrics, Kyorin University School of Medicine, Shinkawa 6-20-2, Mitaka, Tokyo 181-8611, Japan. E-mail: kuniyan@kyorin-u.ac.jp*

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For the last four decades, synthetic glucocorticoids have been widely used and known to have therapeutic effects in resolving proteinuria in various glomerular diseases.[1,2] However, the mechanism by which glucocorticoids govern their therapeutic action against proteinuria remains largely unknown. We have reported that both glucocorticoid-inactivating enzyme (11β-hydroxysteroid dehydrogenase type II) and glucocorticoid receptor (GR) are expressed in the human glomerular podocytes,[3,4] suggesting that podocytes may be target cells for the action of glucocorticoids. The questions then arose as to which molecules in the glomerular podocytes are affected by the action of the glucocorticoids and what is the mechanism of their actions against proteinuria?

Recent studies have shown that disruption of protein trafficking underlies various hereditary and autoimmune diseases.[5,6] Alteration in protein trafficking occurs mainly in the endoplasmic reticulum (ER), which is the central site for folding, post-translational modifications and transport of proteins that are destined to intracellular organelles, plasma membrane or the extracellular space. The intracellular protein trafficking is easily perturbed by ER stress, which can be induced by variety of pathological conditions such as energy depletion, hypoxia, viral infections, and mutations that impair protein folding.[7–11]

Over the last few years, several studies have shown that the expression or distribution of the slit diaphragm protein nephrin is altered in the acquired glomerular injury.[12–16] Among these reports, Kim *et al.*[13] reported on the intracellular accumulation of nephrin in the podocytes of patients with minimal change nephrotic syndrome, suggesting that alteration of the intracellular transport of nephrin in

podocyte may underlie the pathomechanism of the heavy proteinuria in nephrotic syndrome. In a recent study, we showed that when nephrin-expressing cells were treated with the asparagine-linked (*N*-linked) glycosylation inhibitor tunicamycin, the plasma membrane trafficking of nephrin was completely blocked and the unglycosylated nephrin remained in the ER, suggesting the critical role of the *N*-linked glycosylations in nephrin membrane trafficking.[17] Although many reagents including tunicamycin are known to induce ER stress,[7] it seams inadequate to use such non-physiological agent for our present study. Among the physiological ER-stress inducers, glucose is used as an energy source for the synthesis of adenosine 5′ triphosphate (ATP) and also functions as substrate for synthesis of carbohydrate moieties for glycoproteins. Furthermore, ATP is well known to cooperate with lectin-like chaperons in playing a role in the quality control system that regulates glycoprotein folding machinery in the ER.[18] Thus, in the present study, we have adapted a glucose starvation condition as an ER-stress model.[9,19,20]

Several studies have indicated that glucocorticoids may also affect the *N*-linked glycosylation process of glycoproteins.[21–23] In the present study, we have hypothesized that the altered process of nephrin trafficking might be one of the targeting events for the pharmacological effects of glucocorticoids in the acquired glomerular diseases. We show that ER stress caused by glucose starvation perturbs normal *N*-glycosylation of nephrin and inhibits the proper intracellular transport of this molecule to the plasma membrane. More interestingly, we found that dexamethasone (DEX) can partially rescue this disruption in the protein transport resulting in the formation of fully glycosylated nephrin at the plasma membrane. We show that this mechanism is GR mediated and through upregulation of mitochondrial transcripts lead to an increase in the intracellular ATP. To our knowledge, this is the first report on the molecular mechanism of a widely used glucocorticoid against acquired nephrin injury.

RESULTS

Glucose starvation induces ER stress and formation of underglycosylated nephrin

Several immortalized mouse and human podocyte cell lines have been established,[24,25] however, the endogenous expression levels of nephrin in these cell lines are either undetectable by immunofluorescence or not sufficient to perform proper intracellular trafficking studies. Indeed, in our previous report[17] we were unsuccessful to detect the presence of endogenous nephrin using either immunofluorescence or Western blot analysis on an immortalized mouse podocyte cell line. Even after establishing a transfected mouse podocyte cell line with stable expression of a human nephrin cDNA construct,[17] the cell line seemed to gradually loose its recombinant nephrin expression after several passages of cell culturing. Therefore, in the present study, we used a transfected human embryonic kidney (HEK)-293 cell line (293-NPH) stably expressing a full-length construct of human nephrin. The use of stably transfected HEK-293 cells has been repeatedly proved to be both reliable and relevant in nephrin-trafficking studies,[17,26] cell–cell adhesion study[27] as well as signal transduction study of nephrin.[28]

To first examine how rapid glucose starvation may occur in a cell culture, equal numbers of wild-type HEK-293 cells were cultured in mediums containing either 25 or 5.5 mM glucose for 24, 48, and 72 h. It was shown that in the conditioned medium with 25 mM glucose, the concentration of glucose gradually decreased to 8.6 mM during a 72 h period; while in the conditioned medium with 5.5 mM glucose it dropped bellow the detectable limit (0.27 mM) already at 24 h (Figure 1a). Addition of DEX to the 5.5 mM glucose medium did not affect the glucose concentration in the conditioned medium, suggesting that DEX did not alter the glucose metabolism in cells. These data suggested that cells cultured in low glucose medium were exposed to glucose starvation already after 1 day of incubation.

To find out how fast the glucose starvation could trigger an ER stress in cells, we examined the levels of glucose-regulated protein glucose-regulated protein (GRP)78, a key chaperon molecule and indicator of ER stress. As shown in Figure 1b, only in cells cultured in 5.5 mM glucose, the levels of GRP78 was increased in a time-dependent manner, indicating that glucose starvation evoked an ER stress already at 24 h.

This finding tempted us to investigate whether ER stress induced by glucose starvation could also affect the biosynthesis and transport of nephrin. We have previously shown that nephrin isolated from 293-NPH cells migrated as a double immunoband of 185 and 175 kDa, in which the 185-kDa band corresponded to the plasma membrane form and the 175-kDa band appeared to be an ER form of nephrin.[17,26] Nephrin-expressing cells were cultured under the same glucose conditions as described above and their cellular extracts were subjected to Western blot analysis. Figure 2a shows that in cells cultured with 25 mM glucose the abundance of both nephrin forms did not change during 72 h of incubation. However, in cells cultured in low glucose medium, both 185- and 175-kDa forms disappeared in cells cultured over 24 h and only a 155-kDa nephrin form was detected (Figure 2a, lanes 4 and 6). The molecular migration of this nephrin form was clearly different from the aglycoform of nephrin (150 kDa) induced by the *N*-glycosylation inhibitor tunicamycin (last lane in Figure 2a). A reduction in both 185- and 175-kDa nephrin forms was already apparent in cells cultured in 5.5 mM glucose medium incubated for 24 h, most likely due to lower levels of protein synthesis as a response to ER-stress condition.[11] Expression levels of the ER-stress marker GRP78 was dramatically increased in 5.5 mM glucose samples incubated for 48 and 72 h, and the tunicamycin-treated cells, indicating a clear ER stress (Figure 2a, lanes 4, 6, and 7).

In order to determine the reason why the molecular mass of nephrin was decreased under this ER-stress condition, cells

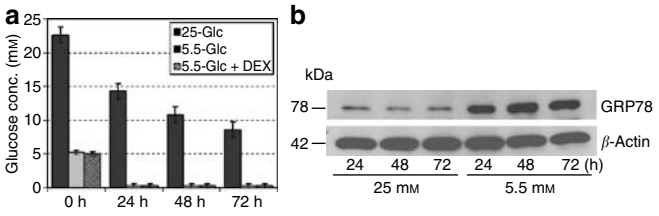

Figure 1 | Rapid consumption of glucose by cells leads to glucose starvation and increased levels of the GRP78 as the ER-stress response. (**a**) Nephrin-expressing 293-NPH cells were cultured in Dulbecco's modified Eagle's medium with 25 mM glucose (25-Glc), 5.5 mM glucose (5.5-Glc), and 5.5 mM glucose medium with 0.1 μM DEX (5.5-Glc + DEX). Glucose concentrations in the conditioned medium were monitored up to 72 h. The measurements were carried out in triplicates and the data represents the means ± s.e.m. (**b**) Time-dependent increased expression of the glucose-regulated protein GRP78 as an ER-stress respond to glucose starvation in cells cultured for 24–72 h. Wild-type HEK-293 cells were cultured in 25 mM or 5.5 mM glucose medium for 24, 48, and 72 h. The expression levels of GRP78 and β-actin as control protein were investigated by Western blot analyses of cellular extracts.

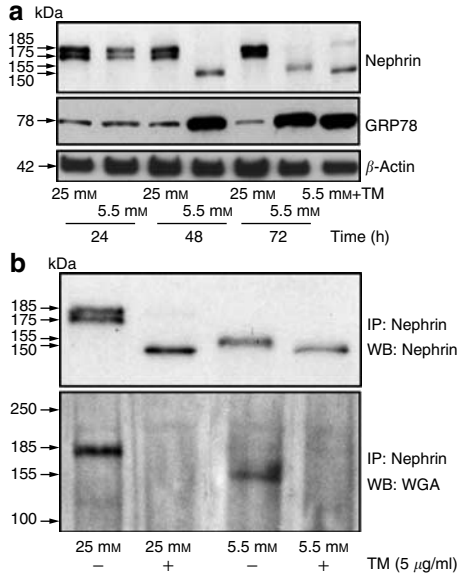

Figure 2 | Induction of underglycosylated nephrin and GRP78 by mild glucose starvation. (**a**) 293-NPH cells were cultured in Dulbecco's modified Eagle's medium medium at the glucose concentrations of 25 or 5.5 mM glucose for the time indicated. Cells were also cultured in 25 mM glucose medium with 5 μg/ml tunicamycin for 24 h. Equal amount of total cellular proteins (10 μg) were separated on 7.5% SDS-PAGE and subjected to Western blot analysis. (**b**) 293-NPH cells were cultured in 25 or 5.5 mM medium in the absence or presence of tunicamycin (5 μg/ml) for 48 h. Cell lysates (500 μg protein) were immunoprecipitated (IP) with anti-nephrin polyclonal antibody and 10 μl aliquots of each were separated on a 5–20% gradient SDS-PAGE gel and immunoblotted with mAb2 mAb against nephrin (upper panel). Same samples were subjected to SDS-PAGE and reacted with the lectin blotting.

were cultured in 25 and 5.5 mM glucose mediums in the absence or presence of tunicamycin for 48 h. Nephrin was immunoprecipitated from cellular extracts and were tested for the presence of carbohydrates by lectin blot analysis using wheat-germ agglutinin (WGA), which recognizes the *N*-acetyl-D-glucosamine and the terminal sugar residue *N*-acetylneuraminic acid (*N*-acetylneuramic acid (sialic acid)) moiety on glycoproteins. Immunoprecipitated nephrin from cells cultured in 25 mM glucose medium was clearly recognized as a broad single band by WGA, suggesting that probably both 185- and 175-kDa glycoform of nephrin were reacted with its terminal sugar *N*-acetyl-D-glucosamine that is the fist building block in the formation of *N*-linked carbohydrates (Figure 2b, lane 1). The 155-kDa nephrin form isolated from cells cultured in 5.5 mM glucose medium was also recognized by WGA, suggesting that this low molecular weight of nephrin contained only *N*-acetyl-D-glucosamine (Figure 2b, lane 3). In contrast, when tunicamycin was included in both mediums, the molecular weight of nephrin was dropped down to 150 kDa, and the WGA blotting showed no reactivity to this aglycoform of nephrin (Figure 2b, lanes 2 and 4), indicating the complete absence of glycosylation. Thus, we concluded that a relatively rapid ER stress could occur as a response to glucose starvation leading to alteration of nephrin *N*-glycosylation in the ER.

Underglycosylated nephrin is retained in the ER

To determine the intracellular localization of underglycosylatd nephrin, cells were cultured in either 25 or 5.5 mM glucose medium for 48 h and were subjected to immunofluorescence and confocal microscopy using anti-nephrin polyclonal antibody and anti-KDEL antibody as ER marker. Cells cultured in 25 mM glucose medium clearly showed the predominant localization of nephrin on the plasma membrane, whereas cells cultured in 5.5 mM glucose medium completely lacked the plasma membrane nephrin form and showed only the intracellular nephrin colocalized with the ER marker (Figure 3a). In order to identify which ER chaperones are predominantly involved in the ER localization of underglycosylated nephrin, we next tested a pull-down experiment with three molecular chaperones in the ER; GRP78, calreticulin and calnexin. Nephrin-expressing cells were cultured in 25 and 5.5 mM glucose medium for 48 h. Calreticulin and calnexin were clearly pull-downed with nephrin in both samples (Figure 3b), while small but detectable amount of GRP78 was pull-downed with nephrin only in the samples from 5.5 mM medium (Figure 3b). The specific binding between calreticulin and nephrin was further confirmed by first pulling down calreticulin and then analyzed the samples with antibody against nephrin. As shown in Figure 3c, the binding of calreticulin to both fully glycosylated and underglycosylated nephrin forms was unambiguously demonstrated. This finding raised the question to as whether calreticulin also binds to nephrin on the plasma membrane. Dual immunostaining of nephrin and calreticulin clearly showed colocalization of both molecules on the cell surface, suggesting broad implication of this chaperone in *N*-glycosylation moiety of nephrin (Figure 3d).

To test the hypothesis that glucocorticoids may govern their protective functions by positively affecting the protein-trafficking process in the ER under cellular stress, we followed the expression and molecular migration of nephrin in glucose-starved cells cultured in 5.5 mM glucose medium either with or without addition of DEX in a dose-dependent manner. Interestingly, we found that 0.1 and 1.0 μM, but not 0.01 μM DEX treatment significantly rescued the formation of 185-kDa form of nephrin compared to that of untreated cells (Figure 4a). In addition, the molecular weights of under-glycosylated nephrin in DEX-treated cells were slightly larger than that of non-treated cells, and more notably, a ladder-like migration of proteins between the 155- and 185-kDa band was seen, clearly indicating the molecular spectrum from the underglycosylated to fully glycosylated nephrin. The reappearance of fully glycosylated 185-kDa nephrin also paralleled the reduction of stress-induced GRP78 protein in the DEX-treated cells, indicating a sign of recovery (lanes 5 and 6 in Figure 4a).

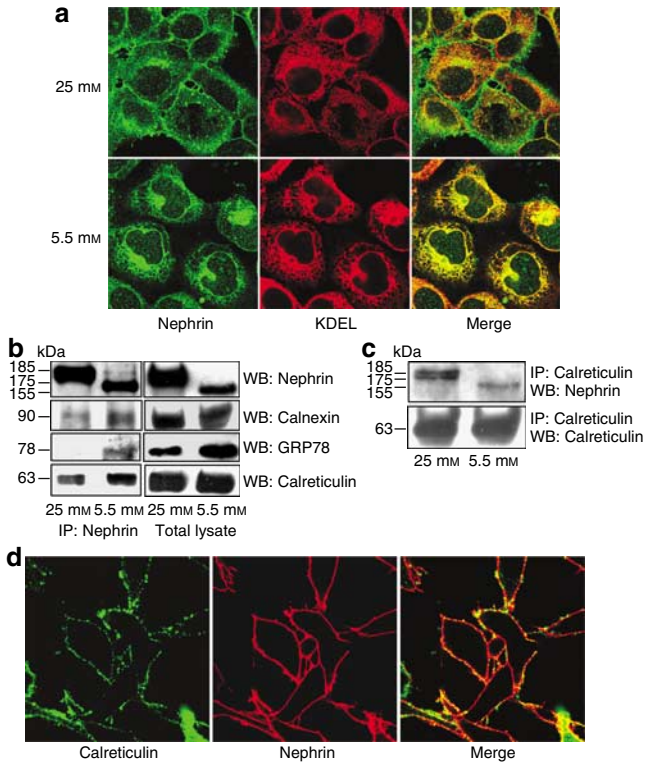

Figure 3 | Underglycosylated nephrin is retained in the ER and bound to lectin-like chaperones. (**a**) Dual immunostaining of nephrin and GRP78/94 (KDEL). 293-NPH cells were cultured in either 25 or 5.5 mM medium for 48 h, subjected to intracellular staining for nephrin (green) and GRP78/94 (red) and observed by confocal microscopy. Nephrin is not present on the plasma membrane of cells cultured in 5.5 mM glucose medium and, instead, is accumulated in the ER compartment. (**b**) and (**c**) Western blotting with nephrin and ER chaperones using immunoprecipitated samples or total lysates from cells as described in Materials and Methods. (**d**) Dual immunostaining of calreticulin and nephrin. Surface immunofluorescence staining was performed in cells cultured in 25 mM glucose medium as described in Materials and Methods.

We next investigated the time-dependent recovery of the mature nephrin form in the glucose-starved 293-NPH cells treated with 0.1 μM DEX. As shown in Figure 4b, the 185-kDa nephrin form could be recovered in cells cultured for 48 and 72 h, clearly indicating a protective role of DEX in the recovery of fully glycosylated nephrin in the ER-stressed cells.

To further investigate whether this protective effect of DEX was receptor mediated through binding to its receptor GR, we used the glucocorticoid antagonist RU486 to block the receptor-mediated effect of DEX. Cells under glucose starvation were pre-treated with 0.01 μM of RU486 for 30 min prior to treatment with increasing DEX concentrations of 0.01, 0.1, and 1.0 μM for 48 h. Western blot analysis showed that when not pre-incubated with RU486, stressed cells responded to the DEX treatment in a concentration-dependent manner with increasing recovery of the fully glycosylated 185-kDa nephrin form (Figure 4c, lanes 4 and 5). However, pre-treatment with RU486 completely abolished the effects of DEX (Figure 4c, lanes 8 and 9), clearly indicating that the observed DEX effect was mediated through binding to its receptor GR. No significant change in the expression levels of GRP78 was seen in samples treated with DEX in combination with RU486.

We also examined surface immunofluorescence staining of cells under the same cell culture conditions. As depicted in Figure 4d, the plasma membrane localization of nephrin was clearly recovered in the glucose-starved cells by treatment with DEX as compared to cells without treatment. This clearly showed that DEX could rescue the mature nephrin form to the plasma membrane. Again the antagonist RU486 abolished this protective role of DEX on the plasma membrane localization of nephrin, confirming the receptor-mediated effect of DEX on rescuing mature nephrin on the cell surface.

DEX increases the intracellular levels of ATP through upregulation of mitochondrial genes

Since the mechanism of chaperon-assisted protein folding in the ER is ATP dependent,[29–31] we next investigated whether glucose starvation *per se* triggered intracellular energy depletion in cells and what could the role of DEX be in this imbalance of energy metabolism. To this end, we monitored the intracellular ATP concentrations in cells cultured either in 25 or 5.5 mM glucose medium for 24, 48, and 72 h. In a parallel experiment, the intracellular levels of ATP was also followed up in cells cultured in 5.5 mM glucose medium treated with 0.1 μM DEX. As shown in Figure 5a, there was no significant difference in the intracellular ATP levels of cells cultured for 24 h in the 25 or 5.5 mM glucose medium either with or without DEX. However, a dramatic decreased to 60 and 55% in the levels of ATP was detected in cells cultured in 5.5 mM glucose medium for 48 and 72 h, respectively. Interestingly, the levels of ATP were brought up to 80% in cells treated with DEX, indicating a significant recovery in the energy depletion in such cells. This observation clearly pointed to the interesting finding that a

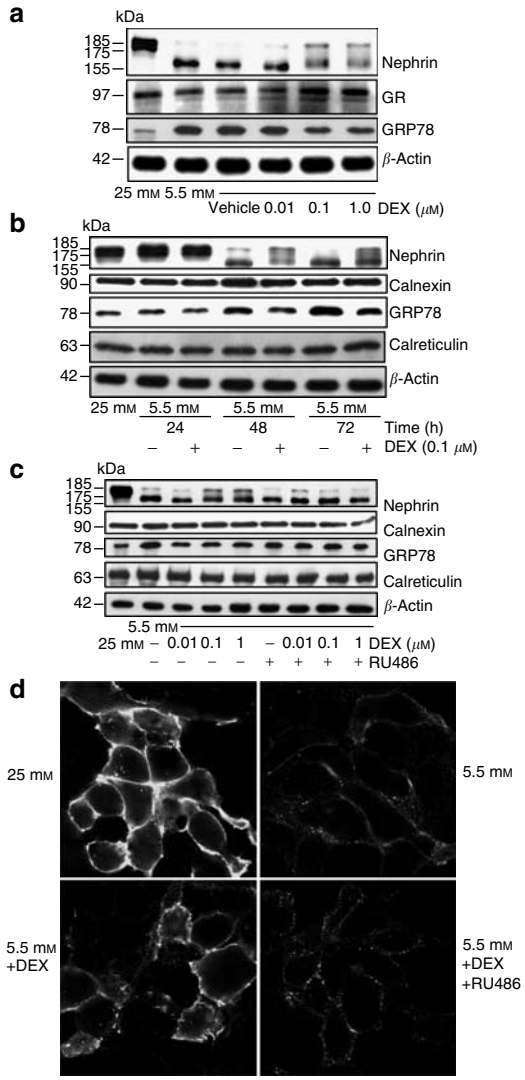

Figure 4 | DEX partially rescues the plasma membrane form of nephrin in the glucose-starved cells through binding to its receptor GR. (**a**) 293-NPH cells were cultured in 5.5 mM glucose medium in the presence of DEX in a dose-dependent manner for 48 h. Cellular extracts of total proteins (10 μg) were subjected to Western blot analysis. DEX rescued 185-kDa nephrin formation at a concentration of 0.1 and 1.0 μM. (**b**) In cells without DEX, 155-kDa nephrin increases in a time-dependent manner. The levels of 185-kDa nephrin were partially recovered by the DEX treatment with concomitant reduction of GRP78. (**c**) Pre-treatment of cells with the glucocorticoid antagonist RU486 at the concentration of 0.01 μM completely abolished the effect of DEX. In cells without RU486 pre-treatment, 185-kDa nephrin was recovered with DEX in a concentration-dependent manner. (**d**) Surface immunofluorescence of nephrin. Cells were cultured in 25 or 5.5 mM glucose medium in combination with DEX and RU486 treatment for 48 h. The antagonist RU486 abolishes the recovery of fully matured nephrin on the cell surface by DEX.

potential mechanism of glucocorticoid actions might be governed by restoring the ATP flux in the ER-stressed cells.

We further compared the expression levels of cytochrome *c* subunit II and ATP synthase, which are components of the membrane-bound protein complexes in the mitochondria involved in ATP production. As shown in Figure 5b, mitochondrial transcripts of cytochrome *c* subunit II and ATP synthase were increase by DEX treatment irrespective of glucose concentration in the medium. Moreover Western blot analysis indicated the similar change of both molecules at a protein level in the samples from isolated mitochondrial fractions (Figure 5c). Although our previous work did not demonstrate the specific existence of GR in the mitochondria of podocyte,[4] these results tempted us to reconfirm its localization in human glomerular podocyte.

It has been recently demonstrated that detergent Triton-X treatment interfere GR labeling of mitochondria.[32] In the present study, immunoelectron microscopy of human podocytes using anti-GR polyclonal antibody and fixation with formaldehyde and acetone without Triton-X treatment clearly showed that in addition to the nucleus and the cytoplasm as previously described, the GR was also localized in the mitochondrial membrane (Figure 5d). This finding further pointed to a dual action of DEX by not only influencing gene expression in the nucleus, but also by localizing into the mitochondria and directly affecting the energy metabolism through gene regulation of mitochondrial DNA.

Glucose and ATP are both needed for proper glycosylation of nephrin

We next tested which one of the two stress conditions, glucose starvation or ATP depletion is underlying the defective glycosylation and intracellular trafficking of nephrin. In order to directly determine a correlation between intracellular ATP level and the amount of mature nephrin, cells were cultured in 25 or 5.5 mM glucose medium in the absence or presence of exogenous ATP for 48 h. They were then simultaneously subjected to the flow cytometry using non-permeabilized cells to compare the amount of surface mature nephrin and to luciferin–luciferase method for the determination of intracellular ATP. As shown in Figure 6a, the ATP level of cells cultured in 5.5 mM glucose medium were estimated as about 70% of that of cells cultured in 25 mM glucose medium. The reduction in ATP levels in these cells was recovered by addition of exogenous ATP in a concentration-dependent manner. Interestingly, as we speculated, the fluorescence intensity of cell surface mature nephrin also drastically increased parallel to elevation levels of intracellular ATP (Figure 6b, a–c). This clearly indicated that ATP alone proved to be a fundamental factor in the recovery of underglycosylated nephrin in the stressed cells.

We next tested the role of glucose in combination with ATP. Three medium conditions were examined; cells cultured in medium with 5.5 mM glucose, cells cultured in medium without glucose, or either of the above mediums supplemented with increasing concentrations of exogenous ATP. Western blot analysis showed that in 5.5 mM glucose medium, the fully glycosylated nephrin could be rescued by addition of exogenous ATP (Figure 6c, lanes 4 and 5). This recovery was concentration dependent as higher levels of ATP increased

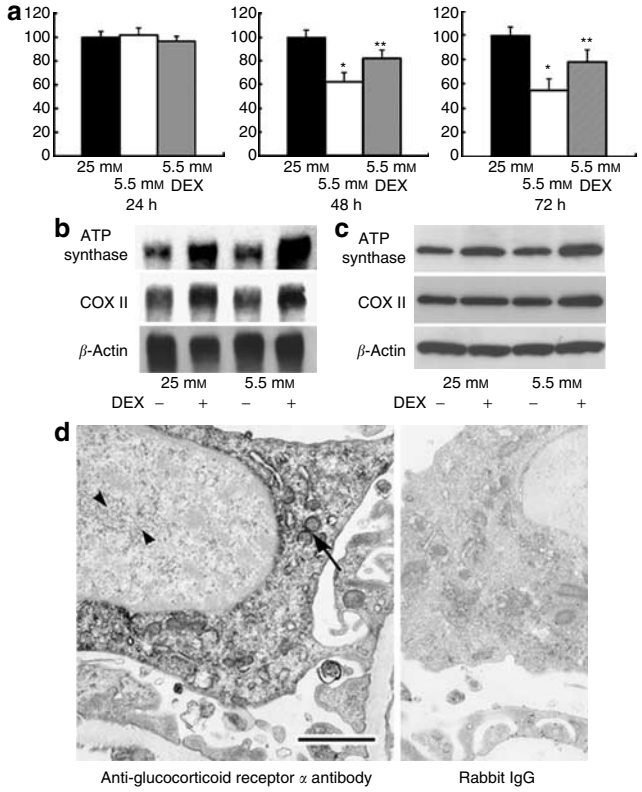

Figure 5 | DEX increases the levels of intracellular ATP. (**a**) Intracellular ATP was measured by bioluminescence detection in 293-NPH cells cultured in 25 or 5.5 mM medium treated with or without 0.1 μM DEX for 24, 48, and 72 h. In all cases, bioluminescence signal (relative light units/μg total protein) was normalized to control (25 mM glucose) as 100%. Histograms show mean normalized values ($\pm$s.e.m) for a sample size $n = 4$. (**b**) Northern blotting with ATP synthase and cytochrome *c* subunit II (COX II). Cells were cultured and treated with DEX as described in Materials and Methods. In all, 2 μg mRNA was separated in agarose gels and hybridized with each digoxigenin-labeled probe. Note apparent increase of ATP synthase and COX II transcripts by DEX treatment both in cells cultured in 25 and 5.5 mM glucose medium. (**c**) Western blotting with ATP synthase and COX II. Cells were cultured in 25 or 5.5 mM medium treated with or without 0.1 μM DEX for 48 h. Mitochondrial fraction was isolated and subjected to SDS-PAGE and reacted with specific antibodies for ATP synthase, COX II and β-actin. Similar positive upregulation of ATP synthase and COX II was seen at a protein level in both stressed and non-stressed cells. (**d**) Pre-embedding immunoelectron microscopy of GR α in human podocytes. Human kidney was fixed with 4%-folmaldehyde followed by cold acetone and reacted with anti-GR α polyclonal antibody (M20) as described in Materials and Methods. GR was detected in the nucleus (arrowheads) and the cytoplasm as we reported in the previous work. In addition, clear localization of GR in the mitochondria membrane (arrows) is also seen. No positive signal was seen in the section treated with rabbit IgG instead of primary antibody. Bar = 2 μm.

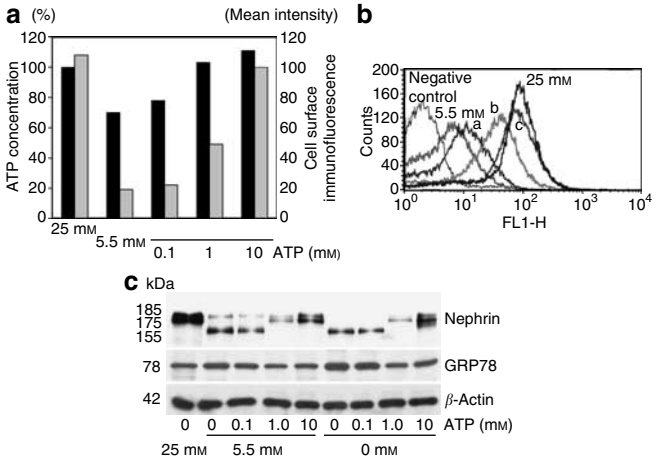

Figure 6 | Direct influence of ATP and glucose on the formation of mature nephrin in stressed cells. (**a**) Correlation between intracellular ATP and surface nephrin expression. Cells were cultured in 25 or 5.5 mM glucose medium in the absence or presence of exogenous ATP. Samples were subjected to ATP assay and flow cytometry, respectively, as described in Materials and Methods. ATP (■) of cells in 25 mM glucose medium was shown as 100% and compared with cells in 5.5 mM medium with or without ATP treatment. The level of surface nephrin expression was calculated as mean fluorescence intensity by flow cytometry (□). (**b**) Actual histogram of flow cytometry with surface nephrin. (**a**) 5.5 mM witout ATP, (**b**) 5.5 + 1.0 mM ATP, and (**c**) 5.5 + 10 mM ATP. (**c**) Addition of ATP compensates for the lower levels but not the lack of glucose. Cells were cultured in 25 mM, 5.5 mM glucose medium or medium without glucose (0 mM) in the presence or absence of ATP for 48 h. Cell extracts were analyzed by Western blot for nephrin, GRP78, and β-actin. In 5.5 mM medium, the 185-kDa form of nephrin is recovered with increasing levels of ATP, whereas in 0 mM glucose medium only the 175-kDa-nephrin form is recovered, indicating that even high levels of ATP cannot compensate for the lack of glucose.

the amount of fully glycosylated nephrin, indicating that the trafficking of nephrin from the ER to the plasma membrane was also increased (Figure 6c, lanes 4 and 5). On the other hand, cells cultured in medium without glucose but supplemented with ATP showed only the 155- and 175-kDa forms of nephrin and were devoid of the 185 kDa fully glycosylated nephrin. Only when the ATP levels were increased to 10 mM some mature 185-kDa nephrin could be seen (Figure 6c, lane 9), suggesting that the presence of glucose is essential for the biosynthesis of fully glycosylated nephrin. The increased levels of GRP78 protein as a stress response to the glucose starvation was, however, reversed to some extend by higher levels of ATP, indicating that ATP can to some degree decrease the ER stress caused by glucose starvation (Figure 6c, lanes 8 and 9). It was concluded that glucose and ATP are both critical for the synthesis of fully glycosylated nephrin.

DEX cannot rescue immature nephrin under ER stress caused by Ca^{2+} imbalance

We finally examined whether DEX may also have a protective role in other ER-stress conditions, such as Ca^{2+} depletion. To this end, cells were cultured in 25 mM glucose medium with or without the presence of A23187 and/or 0.1 μM DEX for 48 h. As was expected, A23187 alone induced a strong expression of GRP78 protein even in the presence of 25 mM glucose concentration in the culture medium. Interestingly, this type of ER stress induced by Ca^{2+} imbalance resulted only in formation of the 175-kDa ER form of nephrin, but not the 155-kDa form (Figure 7). More intriguing, addition

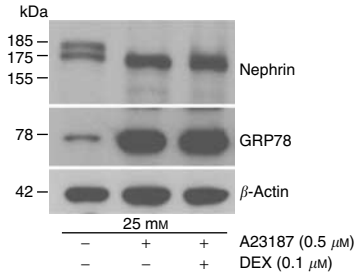

Figure 7 | DEX cannot recover the 185-kDa form of nephrin in cells with Ca^{2+} imbalance. Cells were cultured in 25 mM medium with or without the calcium ionophore A23187 in the absence or presence of DEX. A23187 treatment induced 175-kDa nephrin together with strong induction of GRP78. In contrast to glucose starvation, DEX has no effect on the recovery of the 185-kDa nephrin form in cells with ER stress caused by Ca^{2+} imbalance.

of DEX did not recovered the fully glycosylated 185-kDa form, suggesting a selective pharmacological action of DEX on the ER stress caused by imbalance of energy metabolism but not other stress conditions such as Ca^{2+} imbalance.

DISCUSSION

Human nephrin contains 10 potential *N*-linked glycosylation sites in its extracellular domain where it through homotypic and heterotypic interactions forms the actual ultrafiltration barrier of the glomerulus.[27,33–35] Alteration of such post-translational modification and its potential involvement in the pathomechanism of the proteinuria has not been investigated. Recently, we found that tunicamycin, a potent inhibitor of *N*-glycosylation, completely inhibited the normal plasma membrane localization of nephrin, indicating the crucial role of *N*-glycosylation for the proper trafficking of nephrin from the ER to the plasma membrane.[17] In the present study, we have further investigated the implication of *N*-glycosylation in the intracellular trafficking and cell surface localization of nephrin in an ER-stress condition. Our present study clearly demonstrated that mild glucose starvation induces an ER stress which was independent of tunicamycin-induced pathway,[17] leading to alteration of *N*-glycosylation of nephrin. This resulted in formation of an underglycosylated 155-kDa nephrin form, which retained in the ER and was not transported to the plasma membrane. Lectin blot analysis using WGA identified *N*-acetyl-D-glucosamine as the residual carbohydrate moiety on this underglycosylated nephrin form, indicating that ER stress caused by glucose starvation leads to an early interruption of the biosynthesis of *N*-linked glycosylation of nephrin in the ER.

We further for the first time demonstrated that the ER resident lectin-like chaperons, calreticulin/calnexin most likely played an essential role in the correct folding of nephrin in the ER. Interestingly, calreticulin was also bound to surface mature nephrin. Although a plasma membrane form of calreticulin has been already reported,[36] its functional role on the cell surface remains unclear. However, co-existence of calreticulin and nephrin on the cell surface points to an additional role of this chaperon on the cell surface. On the other hand, the weak interaction of GRP78 and underglycosylated nephrin in the ER-stressed cells may indicate that this chaperone mainly acts as a sensor for unfolded protein response or rather play a role in the circulation of unfolded nephrin in the ER.

Despite their wide application as therapeutic agents against acquired glomerular diseases, the protective mechanisms of glucocorticoids are not well understood. However, in the light of this study, it is possible to consider a potential role of DEX on the energy metabolism in cells under ER-stress condition. It is of great interest that DEX could partially rescue the fully glycosylated nephrin which was properly transported to the plasma membrane. As demonstrated here, the levels of ATP were shown to increase in glucose-starved cells as a response to DEX treatment. Since it has been recently demonstrated that glucocorticoids can directly stimulate the mitochondrial transcripts of cytochrome *c* oxidase subunits II and III as well as cytochrome *c* oxidase activity in the mitochondria,[37,38] we further tested the influence of DEX on the expression of ATP synthase and cytochrome *c* subunit II which both are directly involved in the ATP production. Northern blot analysis showed an obvious upregulation of both genes in the DEX treated cells. In addition, we demonstrated that DEX could stimulate biosynthesis of both molecules also at a protein level by using isolated mitochondrial fraction.

Recent studies have clarified that pretreatment of electron microscopy specimens with Triton-X 100, which is widely used in the conventional immunostaining methods, could abolish mitochondrial labeling of GR.[32] This could explain our previous failure to localize the GR in the pre-embedding immunoelectron microscopy of human podocytes.[4] However, in the present study we could clearly demonstrate the presence of GR in the mitochondria as well as the cytoplasm and the nucleus of human podocytes of kidney specimen which was prefixed with formaldehyde followed by acetone treatment.

Our results suggest that glucocorticoids may exert their protective role in the ER-stressed cells by affecting the intracellular levels of ATP through binding to the receptor GR and localization to both nucleus and mitochondria. Taken together, we propose that DEX could potentially rescue the chaperone function of calreticulin/calnexin through recovery of ATP production by stimulating mitochondrial biogenesis in the ER-stressed cells under energy depletion. However, there are also several other possibilities that could explain the mechanism of DEX in the present study. Although the role of GRP78 in the mechanism by which nephrin is retained in the ER in stressed cells is still unclear, a recent paper has demonstrated that DEX can reduce the GRP78 transcript in the ER-stressed cells induced by chronic hypoxia.[39] Moreover, it has been known that glucocorticoid activates the transcription of glucose-6-phosphate transporter, which translocates glucose-6-phosphate from the cytoplasm to the lumen of the ER, where glucose-6-phosphatase is metabolized to glucose and phosphate.[40] All these possibilities will need further investigation in our model.

MATERIALS AND METHODS

Antibodies and reagents

Mouse monoclonal antibody (mAb) against the ectodomain (mAb2) and rabbit polyclonal antibody against the intracellular domain (pAb2) of human nephrin were previously described.[17,41] Anti-KDEL mouse mAb (SPA-827), against the GRP78/94, anti-calnexin rabbit polyclonal antibody and anti-calreticulin mouse mAb were purchased from StressGen Biotechnologies (Victoria, BC, Canada). The following antibodies were purchased from the suppliers indicated: anti-β-actin mAb (Sigma-Aldrich, St Louis, MO, USA); anti-calreticulin rabbit polyclonal antibody (Affinity BioReagents, Golden CO, USA); anti-GR α rabbit polyclonal antibody, M-20 (Santa Cruz biotechnology, Santa Cruz, CA, USA); anti-OxPhos Complex V subunit α mAb (anti-ATP synthase subunit α), anti-cytochrome *c* oxidase subunit II, Texas Red-X goat anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, and anti-rabbit IgG (Molecular Probes, Eugene, OR, USA); horseradish peroxidase (HRP)-labeled goat anti-mouse and anti-rabbit immunoglobulins (DAKO, Kyoto, Japan). Sodium fluoride, DEX, and RU486 were purchased from Sigma. WGA (EY Laboratories, San Mateo, CA, USA); tunicamycin (Boehringer Mannheim Biochemica, Mannheim, Germany); Complete mini® protein inhibitor cocktail (Roche, Mannheim, Germany) and Protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) were purchased from the suppliers indicated.

Cell culture conditions

The stable cell line of 293-NPH cells expressing a human full-length nephrin construct,[17] was maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 25 mM glucose, supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml Geneticin (Sigma). Once cells had grown to the subconfluent condition, the culture medium was changed to medium containing 25 mM glucose supplemented with 10% charcoal/dextran-treated fetal bovine serum (Perbio, Rockford, IL, USA) for 24 h in order to dissociate endogenous ligands bound to GR and to avoid further GR occupancy by exogenous hormone.[4,42] Subsequently the medium was replaced with either fresh Dulbecco's modified Eagle's medium containing 25 mM glucose or 5.5 mM glucose supplemented with 10% charcoal/dextran-treated fetal bovine serum in the absence or presence of DEX in a time- or a dose-dependent manner. Non-transfected cells were also cultured in 25 or 5.5 mM glucose medium in a time-dependent manner. When a glucocorticoid antagonist was used, cells were pre-incubated with RU486 at a concentration of 0.01 μM for 30 min before adding DEX to the medium. Inhibition of *N*-linked glycosylation by tunicamycin was carried out as described.[17] ATP was added to cells in a dose-dependent manner. Finally, in order to determine whether nephrin glycosylation moieties are also interfered by other ER-stress condition, cells were cultured in 25 mM glucose medium in the absence or presence of DEX (0.1 μM) with A23187 (0.5 μM) for 48 h, which is known to disturb cellular calcium homeostasis and induce cellular ER stress.[7]

Isolation of mitochondrial fraction

Isolation of mitochondrial fraction of 293-NPH cells cultured in 25 or 5.5 mM glucose medium in the absence or presence of DEX (0.1 μM) for 48 h was carried out by using a ApoAlert™ cell fractionation kit (CLONTECH, Palo Alto, CA, USA) according to the manufacturer's instructions.

Immunoprecipitation assay

Cells were washed twice with phosphate-buffered saline (PBS) and lysed on ice in lysis buffer (10 mM Tris-HCl, pH 7.4, containing 1% Triton-X 100, 0.2% saponin, 0.5% NP40, 150 mM NaCl, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, Complete mini®, and 100 μg/ml PMSF). After centrifugation, the supernatants were pre-cleaned with protein A-Sepharose CL-4B (Amersham) for 1 h at 4°C. After centrifugation, the supernatants were incubated with anti-nephrin antibody (pAb2) or polyclonal anti-calreticulin antibody for 1 h at 4°C, and then further incubated with protein A-Sepharose CL-4B for 1 h at 4°C. The pellets were washed four times with lysis buffer, and used for Western blot analysis, or for the reaction with WGA, a lectin specific for *N*-acetyl-D-glucosamine and/or *N*-acetylneuramic acid.

Western and lectin blot analyses

All samples were separated by 7.5 or 5–20% gradient SDS-PAGE under reducing condition. Proteins were transferred to membranes and incubated with blocking buffer (PBS, pH 7.4, containing 2% bovine serum albumin (BSA) and 0.05% Tween-20). The blots were incubated with each primary antibody: anti-nephrin (mAb2: 0.5 μg/ml), anti-GRP78 (KDEL) (1 μg/ml), polyclonal anti-calreticulin (1:2000), anti-calnexin (1:2000), anti-ATP synthase (2 μg/ml), or anti-cytochrome *c* subunit II (2 μg/ml) for 1 h at room temperature. After three series of wash with PBS-containing 0.05% Tween-20, the membranes were incubated for another 1 h with HRP-labeled goat anti-mouse antibody (1:2000) or HRP-labeled goat anti-rabbit antibody (1:2000). Lectin blot analysis was performed using HRP-labeled WGA (5 μg/ml) diluted with 3% BSA in 0.1%-Tween-PBS. All blots were washed twice and signals were detected by using a chemiluminescent kit (PerkinElmer Life Sciences, Boston, MA, USA), according to the manufacturer's instructions. Pre-stained Precision Protein Standard from Bio-Rad (Hercules, CA, USA) was used as molecular standard.

Northern blot analysis

Poly(A)$^+$ RNA was extracted from cells cultured using a mRNA isolation kit (mTRAP, Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Of the poly(A)$^+$ RNA, 2 μg was separated in denatured agarose gel and transferred to nylon membrane (Hybond-N+, Amersham Biosciences) by alkali blotting (3 M NaCl, 0.01 N NaOH). Probes for ATP synthase alpha subunit mRNA (Accession No. BT007209) were prepared by PCR digoxigenin probe synthesis kit (Roche) with the sense primer (5′-GACTGGGACTGCTGAGATGTCC-3′) and the antisense primer (5′-GAGCAACAGAGACATCTGACGG-3′) from ATP synthase alpha subunit cDNA. Probes for human cytochrome *c* subunit II mRNA (Accession No. X15759) were prepared with the sense primer (5′-GGCACATGCAGCGCAAGTAGGT-3′) and the antisense primer (5′-GTAGTATACCCCCGGTCGTGTA-3′) from human cytochrome *c* subunit II cDNA. Probes for human β-actin mRNA (Accession No. NM001101) were prepared with the sense primer (5′-GACAACG GCTCCGGCATGTGCA-3′) and the antisense primer (5′-ATGACC TGGCCGTCAGGCAGCT-3′) from human β-actin cDNA. Each template cDNA for digoxigenin probe synthesis had been prepared with the same primer sets and reverse-transcribed cellular cDNA from the poly(A)$^+$ RNA by PCR amplification under the following conditions: 25 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min. Hybridization and washing were conducted at 65°C. Detection of each mRNA was performed with digoxigenin Luminescent Detection kit (Roche).

Confocal microscopy

Cells were cultured on glass cover slips for 48 h under almost confluent condition in medium with 25 or 5.5 mM glucose in the absence or presence of DEX (0.1 μM) and RU486 (0.01 μM). For surface labeling of nephrin, cells were washed with PBS and fixed in a solution (3% formaldehyde, 4% sucrose in PBS) for 20 min at room temperature. After blocking with 3% BSA in PBS for 1 h, cells were incubated with mAb2 (5 μg/ml) for 1 h at room temperature, and washed and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (10 μg/ml). For the intracellular staining of nephrin, cells were fixed and permeabilized with 0.3% Triton-X 100 in PBS for 10 min. After blocking, cells were incubated with anti-nephrin antibody (pAb2, 1 μg/ml) and anti-GRP78/94 (KDEL) antibody (5 μg/ml) for 1 h at room temperature. The immuno-complex was visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (5 μg/ml) and Texas Red-X goat anti-mouse IgG (5 μg/ml), respectively. Dual staining of nephrin and calreticulin was carried out using mAb2 (5 μg/ml) and anti-calreticulin antibodies (1:100). The immuno-complex was visualized with Texas Red-X goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG, respectively. The cover slips were examined under a confocal laser scanning microscope equipped with a krypton/argon laser.

Immunoelectron microscopy

Human kidney cortex samples were obtained from histologically normal regions of fresh kidneys from patients undergoing nephrectomy with renal cancer or ureter cancer at the Department of Urology, Kyorin University Hospital. Pre-embedding immunoelectron microscopy was performed as described.[3,4] Briefly, kidney cortex samples were fixed in 4% formaldehyde-0.1 M PBS for 6 h at 4°C, immersed in 5, 10, and 20% sucrose PBS, and embedded in 22-oxacalcitriol compound. Frozen sections were further fixed with cold acetone for 20 min. The slides were incubated with blocking buffer (5% goat serum and 3% BSA with PBS) for 1 h and then treated with each primary antibody: anti-GR α (5 μg/ml), rabbit IgG (5 μg/ml) diluted with blocking buffer for 1 h at room temperature, respectively. The slides were incubated with HRP-labeled goat anti-rabbit antibody diluted with blocking buffer, then developed by immersion in 1.4 mM 3,3′-diaminobentizine tetrahydrochloride (Sigma) in PBS. The slides were further treated with 1% osmium tetroxide in 0.1 M phosphate buffer for 10 min, dehydrated by passage through a series of graded ethanol, and embedded in Epon on glass slides. Ultra-thin sections were made, stained with 0.1% lead citrate for 7 min, and examined at 80 kV with a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan).

Bioluminescence detection of intracellular ATP and flow cytometry

Intracellular ATP concentrations were measured by the luciferin–luciferase method using an ATP Monitoring Kit (Roche) under conditions recommended by the manufacturer. The ATP concentration in the extract was then measured with a microplate luminometer (Veritas™, Turner Biosystems, Sunnyvale, CA, USA) and standard ATP solutions. Intensity of mature surface nephrin was measured by flow cytometry using trypsinized cells. Cells were fixed with 3% formaldehyde in PBS for 20 min at room temperature. After washing with PBS, cells were blocked with 3% BSA in PBS for 1 h, followed by the incubation with mAb2 (0.5 μg/ml) for 1 h at 4°C. After washing with PBS, cells were reacted with Alexa Fluor 488-conjugated goat anti-mouse IgG as the secondary antibody, washed and analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) using 488 nm.

Glucose and protein determinations

Glucose concentration of culture medium was determined in triplicate samples using a Glucose kit (HK) from Sigma. Protein concentration was determined by the bicinchoninic acid method according to the procedure described[43] using BSA as the standard.

Data analysis

Statistical significance was determined by an unpaired two-tailed *t*-test. The means $\pm$ s.e.m. for independent experiments are indicated in the figure legends.

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