Mizoribine Corrects Defective Nephrin Biogenesis by Restoring Intracellular Energy Balance

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ABSTRACT
Proteins are modified and folded within the endoplasmic reticulum (ER). When the influx of proteins exceeds the capacity of the ER to handle the load, the ER is “stressed” and protein biogenesis is affected. We have previously shown that the induction of ER stress by ATP depletion in podocytes leads to mislocalization of nephrin and subsequent injury of podocytes. The aim of the present study was to determine whether ER stress is associated with proteinuria in vivo and whether the immunosuppressant mizoribine may exert its antiproteinuric effect by restoring normal nephrin biogenesis. Induction of nephrotic-range proteinuria with puromycin aminonucleoside in mice increased expression of the ER stress marker GRP78 in podocytes, and led to the mislocalization of nephrin to the cytoplasm. In vitro, mizoribine, through a mechanism likely dependent on the inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH) activity in podocytes, restored the intracellular energy balance by increasing levels of ATP and corrected the posttranslational processing of nephrin. Therefore, we speculate that mizoribine may induce remission of proteinuria, at least in part, by restoring the biogenesis of slit diaphragm proteins in injured podocytes. Further understanding of the ER microenvironment may lead to novel approaches to treat diseases in which abnormal handling of proteins plays a role in pathogenesis.


Nephrotic syndrome (NS) is a common disorder with multiple etiologies.1 The pathomechanism of proteinuria in NS is not completely understood, but recent cloning of the gene mutated in the congenital NS, NPHS12,3 and characterization of its protein product, nephrin, opened new avenues for research. Besides its central role in the development of congenital NS, nephrin has been implicated in the pathogenesis of acquired forms of NS.4–7 The role of nephrin in the pathogenesis of NS is not surprising because nephrin is a critical component of the podocyte slit diaphragm, where it is instrumental in maintaining the integrity of the glomerular filtration barrier.8,9 Nephrin is a transmembrane glycoprotein of the Ig superfamily, and, like other glycoproteins, nephrin biogenesis involves steps such as synthesis, folding, modifications, infield of the Ig superfamily, and, like other glycoproteins, nephrin biogenesis involves steps such as synthesis, folding, modifications, impairments of nephrin biogenesis.

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Studies were conducted to elucidate the effect of mizoribine on side (PAN) nephrosis, an present in the rat glomeruli during puromycin aminonucleoside, including nephrin. We verified that ER stress was present in a model of minimal-change NS,4–7 and nephrin biogenesis can be inhibited experimentally during ER stress that is induced by glucose starvation.13 However, ER stress has never been documented during NS in podocytes.

Remission of proteinuria in idiopathic NS can be induced by glucocorticoids with or without immunosuppressive agents. The vast majority of patients with the minimal-change NS achieve complete remission with glucocorticoids alone.15 Additional therapeutic options are available for patients who have minimal-change NS and develop steroid-resistant or frequently relapsing, steroid-dependent NS. These therapeutic options are aimed at the control of NS and at minimizing the exposure to glucocorticoids and include levamisole and alkylating agents such as chlorambucil and cyclophosphamide.16 Other immunosuppressive agents, originally developed for the use in kidney transplantation and more recently used for the treatment of the intractable forms of NS, are cyclosporine, mcyphenolate mofetil, and mizoribine. The mechanism of the antiproteinuric effect of these agents is not completely understood. Compelling evidence, including our recently published data, indicate that some therapeutic agents may exert the antiproteinuric effect, at least in part, by restoring nephrin biogenesis.7,13,17,18

Mizoribine, an agent developed in Japan in 1971 from Eu penicillium brefeldianum, M-2166,19 has been well accepted in Japan for the treatment of steroid-resistant or frequently relapsing, steroid-dependent NS.20 The immunosuppressive effect of mizoribine consists of a selective and competitive inhibition of inosine 5′-monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme in the de novo purine nucleotide biosynthesis21–22 (Figure 1). Because lymphocytes rely solely on the de novo purine biosynthesis, inhibition of IMPDH activity in these cells leads to depletion of the purine nucleotides, inhibition of activated lymphocyte proliferation, and, subsequently, immunosuppression. It is unknown whether remission of proteinuria, induced by mizoribine in susceptible individuals with NS, is mediated exclusively by the effect on activated lymphocytes or whether it may result, at least in part, from a direct effect of mizoribine on the podocyte. Compelling evidence demonstrates that ER stress inhibits the biogenesis of proteins, including nephrin. We verified that ER stress was present in the rat glomeruli during puromycin aminonucleoside (PAN) nephrosis, an in vivo model of minimal-change NS. Studies were conducted to elucidate the effect of mizoribine on nephrin biogenesis during ER stress. We report that mizoribine restored the intracellular energy balance during ER stress by salvaging the ATP levels and rescued the expression of mature, fully glycosylated nephrin in the plasma membrane by a mechanism that depends on the mizoribine-mediated inhibition of IMPDH activity.

**RESULTS**

**ER Stress Is Present in Rat Glomeruli during NS**

Studies were conducted to examine whether ER stress is present in vivo during NS. PAN nephrosis is a well-accepted and widely used in vivo model of minimal-change NS. As demonstrated by the increased expression of the stress-inducible chaperones, glucose-regulated protein 78 (GRP78),23 ER stress was present on days 4 and 5 after injection of PAN, the time that coincides with development of heavy proteinuria in this model of NS (Figure 2). The expression of GRP78 was partially increased in the glomeruli on day 4 of PAN nephrosis, whereas it was globally increased on day 5 (Figure 2B). The pattern of nephrin expression was changed in parallel with the induction of ER stress. In the control glomeruli (day 0), nephrin expression was seen in a linear pattern, as described previously. In contrast, on day 4 of PAN nephrosis, nephrin expression displayed a fine granular and linear pattern in the capillary loops. Moreover, on day 5, the capillary loop pattern was almost completely abolished and a cytoplasmic pattern was prominently observed (Figure 2B). The expression of GRP78 and nephrin on days 1, 2, and 3 after PAN injection was similar to that of the control (data not shown). Taken together, our data demon-
strate altered intracellular localization of nephrin after induction of ER stress in podocytes during PAN nephrosis.

Mizoribine Reduces ER Stress and Corrects Defective Nephrin Biogenesis

Studies were conducted to examine the effect of mizoribine on nephrin biogenesis during ER stress that was induced by glucose starvation. To this end, HEK293 cells stably expressing human nephrin (293-NPH cells) were cultured in medium containing standard (25 mM) or low (5.5 mM) glucose. In the standard glucose medium, nephrin was expressed predominantly as a doublet with molecular weight of approximately 185 and 175 kD, corresponding to the mature, fully glycosylated plasma membrane form and the partially glycosylated ER form of nephrin, respectively, as described previously. Glucose starvation induced ER stress, as demonstrated by increased expression of the stress-inducible chaperone GRP78, and inhibited nephrin biogenesis, as demonstrated by expression of the immature ER form of nephrin with the molecular weight of approximately 155 kD. A 48-h treatment of the glucose-starved cells with MZR reduced expression of GRP78 and corrected defective nephrin biogenesis in a concentration-dependent manner. An equal amount of total cellular proteins (10 µg) was separated by SDS-PAGE using a 7.5% gel. The experiment was repeated three times from separate cultures.

For more direct examination of the effect of mizoribine on nephrin biogenesis, the glucose-starved cells were cultured in the presence of various concentrations of mizoribine for 48 h. Mizoribine reduced expression of GRP78 and corrected defective nephrin biogenesis in a concentration-dependent manner (Figure 3). Moreover, the pattern of nephrin expression in the glucose-starved cells that were treated with 50 µg/ml mizoribine was indistinguishable from that in cells that were grown in the standard glucose medium (Figure 3).

To determine whether ER stress affects the biogenesis of other proteins that are important for the integrity of podocyte foot processes, we examined the effect of glucose starvation on β1-integrin biogenesis. β1-Integrin is expressed in the basal membrane of the podocyte foot processes and plays an important role in the attachment of the podocyte to the glomerular basement membrane through binding to laminin. Similar to nephrin, β1-integrin is a N-glycoprotein and demonstrates dominantly as a 155-kD band (Figure 3), corresponding to an immature ER form of nephrin. For determination of the effect of mizoribine on nephrin biogenesis, the glucose-starved cells were cultured in the presence of various concentrations of mizoribine for 48 h. Mizoribine reduced expression of GRP78 and corrected defective nephrin biogenesis in a concentration-dependent manner (Figure 3). Moreover, the pattern of nephrin expression in the glucose-starved cells that were treated with 50 µg/ml mizoribine was indistinguishable from that in cells that were grown in the standard glucose medium (Figure 3).

For more direct examination of the effect of mizoribine on the expression of nephrin in the plasma membrane, 293-NPH cells were cultured in medium containing standard (25 mM) or low (5.5 mM) glucose in the absence or presence of mizoribine (50 µg/ml for 48 h), followed by surface immunostaining with anti-nephrin antibody, and analyzed by flow cytometry. As illustrated in Figure 4, glucose starvation inhibited nephrin expression in the plasma membrane. Furthermore, mizoribine rescued the plasma membrane expression of nephrin in the glucose-starved cells (Figure 4).
different molecular weights depending on the glycosylation status. Immortalized human podocytes were cultured in RPMI-1640 medium with low glucose (2.2 mM) or standard glucose (11 mM) in the absence or presence of tunicamycin for 48 h. Subsequently, cells were trypsinized, fixed, and stained with a monoclonal anti-nephrin antibody (mAb2) and a secondary Alexa Fluor 488 goat antimouse antibody, and the fluorescence intensities were analyzed, as described in the Concise Methods section.

Figure 4. Summary of flow cytometry experiments demonstrating that MZR partially rescued nephrin expression in the plasma membrane. 293-NPH cells were cultured in medium containing standard (25 mM) or low (5.5 mM) glucose in the absence or presence of MZR (50 μg/ml for 48 h). Subsequently, cells were trypsinized, fixed, and stained with a monoclonal anti-nephrin antibody (mAb2) and a secondary Alexa Fluor 488 goat antimouse antibody, and the fluorescence intensities were analyzed, as described in the Concise Methods section.

Mizoribine Restores Nephrin Biogenesis

The expression of the 120-kD band, most probably a partially glycosylated form of β1-integrin, caused by glucose starvation, indicates that ER stress alters the biogenesis of β1-integrin (Figure 5). Thus, the effect of ER stress on protein biogenesis is not limited to nephrin and may involve other members of the macromolecular slit diaphragm protein complex in podocytes.

Figure 5. Representative Western blot experiment demonstrating that ER stress that was induced by glucose starvation inhibits β1-integrin biogenesis. Human immortalized podocyte cells were cultured either in normal-glucose (11 mM) or low-glucose (2.2 mM) medium in the absence or presence of tunicamycin (5 μg/ml) for 48 h. In podocytes cultured in the standard glucose medium, β1-integrin was expressed predominantly as a 150-kD band, representing a fully glycosylated form of β1-integrin (Figure 5). Tunicamycin induced expression of a 100-kD band, likely an aglycoform of β1-integrin.

Mizoribine Restores the Intracellular Energy Balance during ER Stress

We examined the effect of mizoribine on the intracellular ATP levels during ER stress in 293-NPH cells using HPLC. In glucose-starved cells, the ATP level was significantly decreased compared with cells in the standard glucose medium (Figure 6). These data confirm that glucose starvation caused intracellular energy depletion. Furthermore, treatment with mizoribine at a concentration that rescued nephrin biogenesis (50 μg/ml for 48 h) increased the level of intracellular ATP in the glucose-starved cells (Figure 6). Taken together, these data indicate that the mizoribine-mediated rescue of nephrin biogenesis was mediated by restoration of the intracellular energy balance.

Figure 6. Summary of HPLC experiments performed to determine the effect of MZR on the intracellular ATP levels. 293-NPH cells were cultured in 25 or 5.5 mM glucose medium in the absence or presence of MZR (50 μg/ml for 48 h). Intracellular ATP level was measured by HPLC, as described in the Concise Methods section. In the glucose-starved cells, the ATP level was significantly decreased compared with the cells that were grown in the standard glucose medium. These data confirm that glucose starvation caused intracellular energy depletion. MZR increased the intracellular ATP level in the glucose-starved cells. *P < 0.01. Five measurements were performed per group.
concentrations used in our study, inhibited IMPDH activity. As illustrated in Figure 7, glucose starvation alone had no effect on IMPDH activity in the 293-NPH cells. However, mizoribine (50 μg/ml for 48 h) significantly decreased IMPDH activity in the glucose-starved cells (Figure 7).

If the inhibition of IMPDH activity were responsible, at least in part, for the mizoribine effect on the intracellular energy balance and nephrin biogenesis, then other IMPDH inhibitors would be expected to have a similar effect. To test this prediction, we examined mycophenolic acid (MPA), a metabolite of mycophenolate mofetil and a selective and noncompetitive IMPDH inhibitor that is structurally unrelated to mizoribine.21,26–31 As illustrated in Figure 8, MPA attenuated ER stress, as demonstrated by decreased expression of GRP78, and restored nephrin biogenesis in the glucose-starved 293-NPH cells in a concentration-dependent manner. The pattern of nephrin expression in the glucose-starved cells that were treated with 25 μg/ml MPA was indistinguishable from that in cells that were grown in the standard glucose medium (Figure 8). Taken together, these data indicate that inhibition of IMPDH activity may play a role in the restoration of the intracellular energy balance and rescue of nephrin biogenesis.

Our recently published data demonstrated that rescue of nephrin expression in the plasma membrane by dexamethasone is mediated by the glucocorticoid receptor (GR).13 Mizoribine could interact with the GR through binding to 14-3-3, a protein known to associate with and accelerate the GR function.32 If rescue of nephrin biogenesis by mizoribine were mediated by the GR, then inhibition of the GR would be expected to attenuate this mizoribine effect. As demonstrated in Figure 9, a 30-min pretreatment of the glucose-starved 293-NPH cells

Figure 7. Summary of experiments performed to determine the effect of MZR on IMPDH enzyme activity. MZR is a selective and competitive inhibitor of IMPDH, the rate-limiting enzyme in purine nucleotide biosynthesis. For determination of the effect of MZR on IMPDH activity during ER stress, 293-NPH cells were cultured in 25 or 5.5 mM glucose medium in the absence or presence of MZR (50 μg/ml for 48 h). IMPDH activity was studied by the enzyme activity assay as described in the Concise Methods section. Glucose starvation alone had no effect on IMPDH activity. MZR significantly decreased IMPDH activity in the glucose-starved cells. *P < 0.01. Three experiments were performed per group.

Podocytes Express IMPDH mRNA

These experiments suggest that mizoribine may act directly on IMPDH in podocytes; however, the expression of IMPDH has never been confirmed in podocytes. Two isoforms of IMPDH derived from two different genes, designated type I and II, have been cloned.33,34 For detection of whether these genes indeed exist in the glomerulus and podocytes in vivo, mouse glomeruli were isolated by using magnet beads and further cultured to obtain primary podocytes. As shown

Figure 8. Representative Western blot experiment demonstrating the effect of MPA on nephrin biogenesis. MPA, a metabolite of mycophenolate mofetil, is a selective and noncompetitive IMPDH inhibitor that is structurally unrelated to MZR.21,32–37 Studies were conducted to determine the effect of MPA on nephrin biogenesis. 293-NPH cells were cultured in medium containing standard (25 mM) or low (5.5 mM) glucose. A 48-h treatment with MPA attenuated ER stress, as demonstrated by decreased expression of GRP78 and corrected defective nephrin biogenesis in the glucose-starved cells. An equal amount of total cellular proteins (10 μg) was separated by SDS-PAGE using a 7.5% gel. The experiment was repeated three times from separate cultures.

Figure 9. Representative Western blot experiment performed to determine the effect of the GR inhibitor RU486 on the rescue of nephrin biogenesis by MZR. For determination of whether rescue of nephrin biogenesis by MZR was mediated by the GR, 293-NPH cells cultured in low-glucose (5.5 mM) medium were treatment with the GR antagonist RU486 for 30 min before incubation with MZR (50 μg/ml for 48 h), as described in the Concise Methods section. RU486 did not inhibit the MZR-mediated rescue of nephrin biogenesis. The experiment was repeated three times from separate cultures.

with the GR antagonist RU486 did not interfere with the rescue of nephrin biogenesis by mizoribine. Taken together, these data indicate that mizoribine rescued nephrin biogenesis by a mechanism likely mediated by inhibition of IMPDH activity and independent of the GR.
in Figure 10A, the glomerulus was found to express both transcripts, in which IMPDH I was more abundant compared with the rest of the kidney. Moreover, primary podocytes were found to express distinctly both mRNA (Figure 10B); however, the level of relative expression of IMPDH I was much less than that of IMPDH II. Further experimentation will be required to explore this meaning. Because primary podocytes are difficult to be propagated, we used immortalized murine podocytes to determine whether mizoribine could directly affect IMPDH of podocytes. Differentiated murine podocytes were cultured in RPMI-1640 medium containing a standard amount (11 mM) of glucose, as described previously.35 As illustrated in Figure 11, A and B, IMPDH I and II mRNA were expressed in the podocytes, and their expression was not affected by mizoribine (50 μg/ml for 48 h). Next, studies were conducted to determine the effect of mizoribine on IMPDH mRNA during ER stress. For induction of ER stress, podocytes were cultured in the low-glucose (2.2 mM) medium. Glucose starvation alone upregulated IMPDH I and GRP78 mRNA expression (Figure 11, A and C) but had no effect on the expression of IMPDH II mRNA (Figure 11B). These data indicate that the expression of IMPDH I and II may be differentially regulated in podocytes. These results are in general agreement with the previously published data that in other tissues, the expression of IMPDH I and II is differentially regulated.36 It is interesting that the same mizoribine treatment (50 μg/ml for 48 h), which dramatically reduced the IMPDH enzyme activity (Figure 7), significantly increased the expression of both IMPDH mRNA in the glucose-starved podocytes (Figure 11, A and B). Thus, expression of the IMPDH mRNA may be increased in response to the inhibition of the IMPDH enzyme activity in podocytes. Taken together, these data indicate that the podocyte isoforms of IMPDH may be regulated by mizoribine.

**Glomeruli Express the CNT2 Protein**

These data that mizoribine affects IMPDH in podocytes suggest that mizoribine should be transported into the cells; however, the transport mechanism is unknown. Mizoribine is a water-soluble nucleoside analog structurally similar to ribavirin.31 Recent data demonstrate that the intracellular uptake of ribavirin is mediated by nucleoside transporters (NT).37 Mammalian cells contain two major NT families: The equilibrative, which mediate nucleoside transport in both directions depending on the nucleoside concentration gradient across the plasma membrane, and the concentrative (CNT) ones, which mediate nucleoside transport independent of the nucleoside concentration gradient across the plasma membrane.38,39 The CNT mRNA are expressed in the kidney, and the CNT isofrom 2 (CNT2) mRNA is predominantly expressed in the glomeruli.38,40 However, the glomerular expression and distribution of the CNT2 protein remains unknown. Therefore, studies were conducted to examine the expression of CNT2 in the human glomerulus. To this end, we generated a polyclonal antibody against human CNT2 and characterized its specificity by Western blot analysis using a human glomerular sample. As shown in Figure 12A, anti-CNT2 antibody detected a single approximately 65-kD band corresponding to the predicted molecular weight of CNT2. The immunizing peptide abolished this band, indicating that the anti-CNT2 antibody specifically recognized CNT2 protein in human glomeruli. The positive signal of CNT2 immunostaining was clearly found in the glomerulus (Figure 12B), suggesting that human glomerulus contains a transport mechanism that may be used for the intracellular uptake of mizoribine.

**DISCUSSION**

The major novel observation in this study is that ER stress was induced during NS and that mizoribine, an agent with a known antiproteinuric effect in humans and rats,20,41 reduced ER stress, rescued nephrin biogenesis, and restored the expression of mature, fully glycosylated nephrin in the plasma membrane. These effects of mizoribine were mediated by salvage of the intracellular ATP levels. Our data indicate that inhibition of IMPDH activity by mizoribine plays a role in restoration of the intracellular energy balance and reduction of ER stress. Moreover, our data indicate that mizoribine may act directly on podocytes and that these cells contain a transport mechanism that may be used for the intracellular uptake of mizoribine.

Compelling evidence highlights the critical role of podocyte injury in the pathogenesis of proteinuria and NS.22,43 Podocyte injury, similar to the injury of other cells, is closely associated with ER stress.44–47 In this study, we demonstrated for the first time the presence of ER stress in the rat glomeruli during NS induced by PAN. Furthermore, we demonstrated that nephrin distribution was altered from the normal capillary loop pattern to a cytoplasmic pattern, at the time of increased expression of GRP78 and heavy proteinuria during PAN-induced nephrosis. Similar redistribution of nephrin into the cytoplasm of podocyte during minimal-change NS was also found in the previously published work.48,49 Thus, ER stress may play a critical role in the pathogenesis of proteinuria and NS.
role in the pathomechanism of proteinuria in minimal-change NS. Although we did not examine in this study whether mizoribine exerts the antiproteinuric effect on PAN nephrosis, Shibasaki et al. already demonstrated the antiproteinuric effect of mizoribine.

To examine the mechanism of the antiproteinuric effect of mizoribine in vitro, we used a previously characterized model whereby nephrin biogenesis was inhibited by the intracellular energy depletion. In this model, glucose starvation of cultured cells leads to depletion of the intracellular ATP levels by suppressing the mitochondrial oxidative phosphorylation and by inhibiting the synthesis of substrates for the de novo pathway of purine nucleotide (ATP and GTP) biosynthesis. During intracellular energy depletion, induction of ER stress was demonstrated by increased expression of the stress-inducible chaperone GRP78, and the inhibition of nephrin biogenesis was confirmed by expression of the immature, 155-kD nephrin (Figure 3). Under these experimental conditions, mizoribine rescued nephrin biogenesis, as demonstrated by expression of the mature, fully glycosylated, 185-kD form of nephrin and by the rescue of nephrin targeting to the plasma membrane.

Data presented in this article indicate that ER stress may have a more global effect on protein biogenesis in podocytes because ER stress also inhibited the biogenesis of another N-glycoprotein, β1-integrin. Studies beyond the scope of this article are needed to determine whether ER stress affects the biogenesis of additional proteins, besides nephrin and β1-integrin.

Figure 11. Summary of reverse transcriptase–PCR experiments performed to determine the expression of IMPDH I and IMPDH II mRNA in differentiated immortalized mouse podocytes. Differentiated, immortalized mouse podocytes were cultured in medium containing standard (11 mM) or low (2.2 mM) glucose in the absence or presence of MZR (50 μg/ml for 48 h). Total RNA (1 μg) was amplified using specific primer sets for IMPDH I (A), IMPDH II (B), and GRP78 (C) as described in the Concise Methods section. The mRNA were normalized to the β-actin mRNA and expressed as fractions of the corresponding mRNA from cells that were cultured in standard glucose medium without MZR. In cells that were cultured in the standard amount of glucose, MZR did not affect the expression of IMPDH I or II. Glucose starvation alone increased expression of IMPDH I but had no effect on the expression of IMPDH II, suggesting that expression of IMPDH I and II may be differentially regulated in podocytes. MZR increased expression of both IMPDH mRNA in the glucose-starved podocytes. *P < 0.01; **P < 0.05. Five experiments were performed in each group.

Our data demonstrate that mizoribine may rescue protein biogenesis by salvaging the intracellular ATP levels. Several lines of evidence indicate that recovery of intracellular ATP levels in the glucose-starved cells by mizoribine was mediated by inhibition of the activity of IMPDH, the rate-limiting enzyme in the purine nucleotide biosynthesis. First, at concentrations used in our study, mizoribine inhibited IMPDH activity. Second, rescue of intracellular ATP in the glucose-starved cells was also achieved by MPA, another IMPDH inhibitor structurally unrelated to mizoribine. Moreover, the mechanism of the ATP rescue by mizoribine was independent of the GR and therefore different from that of the glucocorticoids. Because of the existing interconnections between the ATP and GTP biosynthetic pathways (Figure 1), it is conceivable that the mizoribine-mediated inhibition of IMPDH activity could salvage the intracellular ATP levels by shunting the IMPDH substrate (IMP), depleted during glucose starvation, from the GTP to the ATP biosynthetic arm. Additional studies beyond the scope of this work are required to identify the exact mechanism.

Our data suggest that the remission of proteinuria induced by mizoribine in NS may be mediated, at least in part, by a direct effect on podocytes because (1) glomeruli contain a transport system that may be used for the intracellular uptake of mizoribine, as demonstrated by the expression of CNT2,
and (2) podocytes express the IMPDH transcripts, which are regulated by mizoribine during ER stress. Moreover, the effects of mizoribine on the intracellular energy balance and nephrin biogenesis were observed at clinically attainable concentrations. A better understanding of the pathomechanism of NS and the mechanisms by which antiproteinuric agents lead to remission in NS may allow development of new therapies.

**CONCISE METHODS**

**Cell Culture**

HEK293 cells stably expressing full-length human nephrin (293-NPH) were cultured in DMEM (Invitrogen, Carlsbad, CA) containing standard (25 mM) glucose as described previously. Con-
laser-scanning microscope equipped with a Krypton/Argon laser (MRC1024; Bio-Rad Laboratories, Hercules, CA).

**Determination of the Effects of Mizoribine on Nephrin Biogenesis during ER Stress Induced by Glucose Starvation**

Subconfluent 293-NPH cells were cultured in DMEM containing 25 mM glucose supplemented with the charcoal/dextran-treated 10% FBS (Perbio, Rockford, IL) for 24 h as described previously. Subsequently, cells were cultured in medium containing standard (25 mM) or low (5.5 mM) glucose in the absence or presence of various concentrations of mizoribine for 48 h. For determination of whether the effects of mizoribine were mediated by the GR, cells were preincubated with the 0.01 μM RU486, a GR antagonist, for 30 min before the mizoribine treatment. Cellular proteins were obtained by ice-cold lysis buffer and subjected to Western blotting with nephrin, GRP78, and β-actin.

In additional experiments, immortalized human podocytes were cultured in RPMI-1640 medium containing standard (11 mM) or low (2.2 mM) glucose for 48 h in the absence or presence of N-glycosylation inhibitor tunicamycin (5 μg/ml). Cellular lysates were subjected to Western blotting with β1-integrin.

**IMPDH Activity Assay**

IMPDH activity assay was carried essentially as described previously with the following modifications. 293-NPH cells were cultured in DMEM containing 25 or 5.5 mM glucose in the absence or presence of 50 μg/ml mizoribine for 48 h at 37°C. Cells were washed with PBS at room temperature and incubated with ice-cold lysis buffer. After an incubation at 4°C for 1 h, cells were homogenized and centrifuged at 600 × g for 10 min at 4°C. The supernatant was centrifuged at 10,000 × g for 10 min at 4°C, and the resultant supernatant was further centrifuged at 10,000 × g for 20 min at 4°C. The reaction mixture consisted of 1 mM IMP, 0.5 mM NAD+, 100 mM KCl, and 40 mM K+ phosphate buffer (pH 7.4). The specific IMPDH activity was expressed in nmol/mg protein per h. The protein was determined using BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) with BSA as the standard.

**Reverse Transcriptase–PCR**

Glomeruli were isolated from adult ICR mice by means of the magnet beads perfusion method. Total RNA was obtained from both of these glomeruli and the rest of the kidney. Primary podocyte culture was further performed using these isolated glomeruli and subjected to the isolation of total RNA as previously reported. Differentiated immortalized mouse podocytes were cultured in RPMI-1640 medium containing either 11 or 2.2 mM glucose in the absence or presence of mizoribine. The reverse transcriptase–PCR for mouse inosine 5'-monophosphate dehydrogenase-I (IMPDH-I) was carried out using the sense primer 5'-GTGGGTGATGTCTGTGAGG-3' and the antisense primer 5'-GCAATGGAAACACACACCCA-3'. Mouse IMPDH-II was carried out using the sense primer 5'-CCCCATTGTTTCTCTACAGC-3' and the antisense primer 5'-ACATCCACACGCAAAGGC-3'. Mouse GRP78 (Accession No. D78645) was carried out using the sense primer 5'-GAAGTTCACTGTGGTGGCGG-3' and the antisense primer 5'-ATCTCCATTAGTGCCACCC-3'. One microgram of total RNA was amplified under the following conditions: 25 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The PCR products were analyzed by electrophoresis on 1% agarose gels, followed by the direct sequence analysis to confirm its correct product.

**Determination of ATP Level by HPLC**

293-NPH cells were harvested with a rubber policeman and pelleted by centrifugation at 600 × g for 10 min at 4°C. The cellular pellet was suspended in perchloric acid (final at approximately 0.2 N) and thoroughly disrupted with a Hitachi HG30 homogenizer (Hitachi Co., Tokyo, Japan) at the maximal speed for 40 s. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The resultant supernatant was neutralized with 2 M KHCO3 (0.4 vol/vol), left on ice for 10 min, and centrifuged at 10,000 × g for 15 min. The resultant supernatant was filtered with the Millex-LH filter (Millipore Japan, Tokyo, Japan) and stored at −20°C until analysis. HPLC was performed using a Supelcosil LC-18T column equipped with the Shimadzu LC-10AD chromatography system (Shimadzu Corp., Kyoto, Japan). 8-Bromo ATP was added to the sample as the internal standard. Nucleotides were identified from their absorption spectra by a diode array spectrophotometer (Shimadzu SPD-M10AVP, Kyoto, Japan).

**Flow Cytometry**

Studies were conducted to determine the effects of mizoribine on the plasma membrane expression of nephrin by flow cytometry, as described previously. Briefly, 293-NPH cells were cultured in DMEM containing 25 or 5.5 mM glucose in the presence or absence of 50 μg/ml mizoribine for 48 h. After trypsinization, cells were fixed with 3% formaldehyde, washed with PBS, and blocked with 3% BSA. Subsequently, cells were incubated with a monoclonal anti-nephrin antibody (0.5 μg/ml) at 4°C for 60 min, washed, and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody. Fluorescence intensities were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) using 488 nm.

**Statistical Analysis**

Statistical analysis of the data was performed using StatView software (Stat Corp., College Station, TX). Means were compared using a two-tailed t test. P < 0.05 was considered significant. Data are expressed as means ± SE.

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**DISCLOSURES**

None.
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