Pex11a Deficiency Is Associated With a Reduced Abundance of Functional Peroxisomes and Aggravated Renal Interstitial Lesions

Huachun Weng, Xu Ji, Kosuke Endo, Naoharu Iwai

Abstract—Although proteinuria is known to be associated with the deterioration of chronic kidney disease, the molecular basis of this mechanism is not fully understood. We previously found that Pex11a deficiency was associated with a reduction of functional peroxisomes and impaired fatty acid metabolism in hepatocytes and resulted in steatosis. Proximal tubule cells are rich in peroxisomes. We assessed whether Pex11a deficiency might result in the derangement of peroxisome systems in proximal tubule cells and the aggravation of tubulointerstitial lesions in chronic kidney disease. Histological analyses showed that the number of functional peroxisomes in proximal tubule cells was reduced in Pex11a knockout (Pex11a−/−) mice. To clarify whether a decrease in the number of tubular peroxisomes might aggravate interstitial lesions, we assessed 2 models in which proximal tubule cells are overloaded with fatty acids (ie, deoxycorticosterone acetate and salt hypertension and the overload of fatty acid–bound albumin). Deoxycorticosterone acetate–salt–treated Pex11a−/− mice exhibited greater interstitial lesions than deoxycorticosterone acetate–salt–treated wild-type mice in terms of tubular lipid accumulation, blood pressure, urinary albumin, urinary N-acetyl-β-d-glucosaminidase, urinary 8-iso-prostan, and the histological evaluation of fibrosis and inflammation. An overload of fatty acid–bound albumin also resulted in more severe tubulointerstitial lesions in Pex11a−/− mice than in wild-type mice. Fenofibrate, a peroxisome proliferator–activated receptor-α agonist, restored the abundance of peroxisomes and reduced the tubulointerstitial lesions induced by deoxycorticosterone acetate–salt hypertension. In conclusion, our results indicate that proximal tubule peroxisomes play an important role in proteinuria-induced interstitial lesions. The activation of tubular peroxisomes might be an excellent therapeutic strategy against chronic kidney disease. (Hypertension. 2014;64:00-00.) ● Online Data Supplement

Key Words: fatty acid–binding proteins ▪ hypertension ▪ peroxisomes ▪ renal insufficiency, chronic

Chronic kidney disease (CKD) is a major risk factor for various cardiovascular diseases. CKD is a cause of proteinuria, and proteinuria is well known to be an aggravating factor for CKD. However, the molecular basis of this proteinuria-induced deterioration of kidney function remains unclear. Most proteins, including albumin, filtered in glomeruli are reabsorbed by proximal tubule cells. Usually, albumin is bound to free fatty acids (FFAs; especially long-chain fatty acids). Reabsorbed FFA-bound albumin has been demonstrated to mediate tubulointerstitial lesions in proteinuric kidney disease. Furthermore, FFAs, rather than albumin, have been shown to be an important factor that leads to tubulointerstitial lesions because of massive albuminuria.

Peroxisomes are ubiquitous organelles bound by a single membrane, which have many important metabolic functions, including β-oxidation of long-chain fatty acids, α- and β-oxidation of long-branched-chain fatty acids, synthesis of cholesterol and ether lipids, and H2O2 metabolism. Peroxisomes are highly versatile and dynamic organelles whose size, shape, number, and protein content vary according to the cell type, metabolic requirements, and extracellular stimuli. Two types of peroxisome biogenesis have been established: budding from the endoplasmic reticulum (ER) and multiplication through elongation and fission. The Pex11 gene family is involved in peroxisome elongation, an initial step in peroxisome fission, and and 3 subtypes (a, b, and c) have been identified in mammals. Pex11a and Pex11c are tissue specific and expressed most prominently in the kidney and liver, whereas Pex11b is expressed ubiquitously. Our previous studies suggested that Pex11a-deficiency–impaired peroxisome elongation and resulted in a reduction of functional peroxisomes, which finally resulted in a reduced capacity of fatty acid oxidation and steatosis formation. The purpose of the present study was to assess the hypothesis that Pex11a knockout (Pex11a−/−) mice might be susceptible to the overloading of fatty acids to proximal tubule cells. We investigated 2 models of kidney disease (ie, deoxycorticosterone acetate–salt [DOCA-salt]) hypertensive kidney disease and FFA-bound BSA-overload nephropathy.
Methods
Experimental details and any associated references are available in the online-only Data Supplement.

Results
Pex11a Deficiency Affects Peroxisome Abundance and Morphology in Tubule Cells
Megalin was used to determine the location of proximal tubules,23 and peroxisome membrane protein 70 (PMP70), localized in the membranes of peroxisomes,24 was used to identify the peroxisomes (Figure 1A). Double-immunofluorescence analysis showed that the number of peroxisomes in the proximal tubules of Pex11a−/− mice was significantly lower than that in wild-type (WT) mice under the basal condition (Figure 1B; P<0.05).

Irregular or elongated spheromes (Figure 1A, B; roundness ≥1.14, ie, categories V–VIII, where a larger roundness score reflects a more elongated shape) were more prevalent in WT mice than in Pex11a−/− mice under the basal condition (Figure 2B; P<0.05).

Influence of DOCA-Salt on Peroxisome Abundance
After 8 days of treatment with DOCA-salt, the number of peroxisomes was significantly reduced in both WT and Pex11a−/− mice (Figure 1B). Treatment with DOCA-salt also downregulated the mRNA expression level of Pex11a in WT mice, which was restored by treatment with fenofibrate (Figure 1C).

Pex11a Deficiency Aggravates Interstitial Lesions Caused by the Treatment With DOCA-Salt
The daily mean systolic blood pressure (as assessed by telemetry) in Pex11a−/− mice was significantly higher than that in WT mice from the third day of DOCA-salt treatment (Figure 3A; P<0.05). Twenty-four–hour urinary albumin (Figure 3B; P<0.01 on day 8) and N-acetyl-β-d-glucosaminidase levels (Figure 3C; P<0.01 on day 3) in Pex11a−/− mice were significantly higher than those in WT mice.

Oil Red O staining revealed that treatment with DOCA-salt obviously increased lipid accumulation in tubule cells in both Pex11a−/− and WT mice. Furthermore, lipid accumulation seemed to be exaggerated in Pex11a−/− mice (Figure S1, middle in the online-only Data Supplement).

The cortical area of Pex11a−/− mice showed more severe tubulointerstitial lesions after treatment with DOCA-salt (Figure 4A). The areas of tubulointerstitial injury (Figure 4C; P<0.01) and fibrosis (Figure 4D; P<0.01) in Pex11a−/− mice were significantly greater than those in WT mice.

Pex11a−/− mice showed a greater extent of macrophage infiltration than WT mice (Figure S2A and S2C) under treatment with DOCA-salt.

Pex11a Deficiency Aggravates Renal Lesions, Fibrosis, and Macrophage Infiltration Induced by Overload of FFA-Bound Albumin
Although this model might be artificial, no significant difference in blood pressure was observed between WT mice and Pex11a−/− mice (Figure S3). After overloading with FFA-bound albumin, Oil Red O staining revealed that Pex11a−/− mice showed more obvious lipid accumulation than WT mice (Figure S4). Overloaded fatty acids resulted in more pronounced tubulointerstitial lesions in Pex11a−/− mice (Figure 4B). Tubulointerstitial injury, fibrosis, and macrophage infiltration in the cortex of Pex11a−/− mice were significantly greater than those in WT mice (Figure 4E and 4F; Figure S2D). Furthermore, Pex11a−/− mice showed higher 24-hour urinary N-acetyl-β-d-glucosaminidase excretion than WT mice (Figure S2E). Overloaded fatty acids significantly decreased the abundance of peroxisomes in both WT mice and Pex11a−/− mice, and the number of functional peroxisomes in Pex11a−/− mice was significantly lower than that in WT mice before and after FFA-bound albumin overload (Figures S5 and S6).

Figure 1. Peroxisome abundance and Pex11a gene expression in the kidneys of deoxycorticosterone acetate (DOCA)-salt–treated mice. 
A. Representative immunofluorescence images with staining for megalin (red), a protein that is highly expressed in proximal tubule cells, which was used to identify proximal tubule cells, and peroxisome membrane protein 70 (green), which was used to identify peroxisomes. 
B. The number of peroxisomes was counted with Image J software in A. Multiple regression analysis indicated that the number of peroxisomes were significantly determined (P<0.0001) by strains (wild-type [WT] versus Pex11a−/−; P=0.0016), DOCA-salt treatment (P<0.0001), and fenofibrate treatment (P<0.001). Subsequently, differences among groups were assessed by ANOVA followed by Turkey Honestly Significant Difference (HSD) test. 
C. Pex11a mRNA levels were assessed in the kidneys. *P<0.05, **P<0.01 by ANOVA followed by Tukey HSD test. DOCA and DOCA-Feno represent DOCA-salt–treated and DOCA-salt and fenofibrate-treated conditions. Scale bar, 10 μm. Error bars, mean±SD.
Pex11a-Deficiency–Impaired Antioxidant Capacity and Increased Reactive Oxygen Species

We performed double-immunostaining with catalase (a peroxisomal matrix protein) and PMP70 (a peroxisomal integral membrane protein; Figure 5A). Although the difference in peroxisome abundance between WT mice and Pex11a−/− mice after treatment with DOCA-salt was not significant (Figure 1B), Pex11a−/− mice exhibited fewer functional peroxisomes (contained both PMP70 and catalase) and a higher proportion of empty peroxisomes (contained PMP70 but not catalase) than WT mice under basal, DOCA-salt, and combined DOCA-salt and fenofibrate conditions (Figure 5B and 5C). Urinary 8-iso-prostanate levels (Figure 5D; P<0.01) and renal reactive oxygen species (ROS) levels (Figure 7; dihydrothidium staining) in DOCA-salt–treated Pex11a−/− mice were higher than those in DOCA-salt–treated WT mice.

Effects of Fenofibrate on Tubulointerstitial Lesions

Fenofibrate restored the decrease in the number of peroxisomes in both DOCA-salt–treated WT mice and DOCA-salt–treated Pex11a−/− mice (Figure 1). The peroxisomes became more round in DOCA-salt–treated WT mice, but this was restored by treatment with fenofibrate (Figure S8). Lipid accumulation in tubular cells (Figure S1), urinary albumin, N-acetyl-β-D-glucosaminidase (Figure 3B and 3C), and 8-iso-prostanate excretion (Figure 5D), renal ROS levels (Figure 7), the tubulointerstitial lesion score and fibrosis, and macrophage infiltration (Figure 4; Figure S2C) were all attenuated by treatment with fenofibrate. Moreover, fenofibrate attenuated an increase in systolic blood pressure induced by DOCA-salt in both WT mice and Pex11a−/− mice (Figure 3D; as assessed by telemetry). However, the levels of tubulointerstitial lesions, macrophage infiltration, and renal ROS in Pex11a−/− mice were still significantly higher than those in WT mice.

Discussion

In the present study, we found that Pex11a deficiency was associated with a reduction in the abundance of functional peroxisomes in proximal tubule cells, and aggravated tubulointerstitial lesions in DOCA-salt hypertension and FFA-bound albumin-overload models, possibly through lipid toxicity. Furthermore, the induction of peroxisome abundance by fenofibrate significantly attenuated the interstitial lesions. Our hypothesis is shown in Figure 6.

The number of peroxisomes might be determined by 2 factors: the biogenesis of peroxisomes and the degradation of peroxisomes (pexophagy). Recent biochemical and morphological studies have revealed 2 mechanisms for peroxisome biogenesis. One mechanism is multiplication through the growth and division of pre-existing peroxisomes (elongation, segregation and constriction of the peroxisomal membrane, and division). There was a greater prevalence of regular spherically shaped peroxisomes in the proximal tubule cells of Pex11a−/− mice under the control condition (Figure 2), indicating that Pex11a-deficiency–impaired peroxisome elongation. Intriguingly, DOCA-salt hypertension reduced the abundance of peroxisomes in WT mice (Figure 1). One of the reasons for this reduction might be the downregulation of Pex11a because the number of elongated peroxisomes decreased and biogenesis from elongation and fission seemed to be reduced (Figure S8). However, overexpression of the hspPEX11a gene in human embryonic kidney cells is associated with elongated peroxisomes,[19] indicating that the induction of Pex11a may improve peroxisome biogenesis. Indeed, treatment with fenofibrate restored the decreased Pex11a expression and the reduction of elongated peroxisomes induced by DOCA-salt and then increased the abundance of peroxisomes.

Fenofibrate also restored the decreased abundance of peroxisomes in DOCA-salt–treated Pex11a−/− mice, indicating that there may be some mechanisms that do not involve Pex11a, such as budding from ER. Budding from ER is another mechanism for peroxisome biogenesis[15-18]. However, a new peroxisome budding from ER requires a maturation process, in which matrix proteins, such as catalase, are imported through peroxisomal targeting signal 1 or peroxisomal targeting signal 2. PMP70 seems to exist on newly synthesized peroxisomes that are just budding.
However, catalase is gradually imported to the newly synthesized peroxisomes. It is highly likely that peroxisomes that exhibit the presence of PMP70 and the absence of catalase are on the path to maturation (dysfunctional peroxisomes). The absence of catalase might indicate that other matrix proteins are also being imported. Double-immunofluorescence analysis showed that the number of functional peroxisomes, as defined by the presence of both PMP70 and catalase, was lower in Pex11a−/− mice (Figure 3C), indicating that peroxisome biogenesis via budding from ER may be substantial in Pex11a−/− mice when compared with WT mice. Budding from ER has slower kinetics and could not be effective or timely for protecting against extracellular stimuli, as well as the elongation of functional peroxisomes, as defined by the presence of both PMP70 and catalase, was lower in Pex11a−/− mice (Figure 3C), indicating that peroxisome biogenesis via budding from ER may be substantial in Pex11a−/− mice when compared with WT mice. Budding from ER has slower kinetics and could not be effective or timely for protecting against extracellular stimuli, as well as the elongation

Figure 3. Pex11a deficiency aggravates deoxycorticosterone acetate (DOCA)-salt-induced hypertension, albuminuria, and urinary N-acetyl-β-D-glucosaminidase (NAG). A, Systolic blood pressure (SBP) in DOCA-salt hypertension mice was monitored by telemetry. Urinary albuminuria (B) and NAG (C) levels were elevated by treatment with DOCA, and this increase was prevented by treatment with 0.05% fenofibrate (Feno, a peroxisome proliferator-activated receptor-α agonist; wt/wt). D, These experiments were performed independently from A to assess the effect of fenofibrate on SBP induced by treatment with DOCA-salt. The percentage increase in SBP was calculated as follows: $\%\text{increase in SBP} = \frac{\text{SBP}_t - \text{SBP}_0}{\text{SBP}_0} \times 100$. **P<0.01 vs DOCA-WT; ##P<0.01 vs DOCA-Feno-WT; or DOCA-Feno-Pex11a−/− by a multivariate ANOVA (MANOVA) followed by Tukey Honestly Significant Difference test. Error bars, mean±SD. WT indicates wild-type.

Figure 4. Pex11a deficiency aggravates tubulointerstitial lesions. A and B, Representative images of Masson Trichrome-stained kidney sections of mice under basal, deoxycorticosterone acetate (DOCA)-salt–treated, DOCA-salt and 0.05% fenofibrate-treated (DOCA-Feno), and free fatty acid–bound albumin (BSA) overload conditions. DOCA or BSA-treated Pex11a−/− mice showed more severe tubular dilatation/atrophy (*), tubule vacuole formation (arrows), epithelial cell necrosis, and interstitial edema and fibrosis (blue). The area of tubulointerstitial injury or fibrosis, as well as the entire cortical area, in 10 high-power fields was measured with Image J software. The degree of tubulointerstitial injury (C and E) and fibrosis (D and F) was evaluated as a ratio relative to the entire cortical area. Multiple regression analysis indicated that tubulointerstitial injury was determined by strain (wild-type [WT] vs Pex11a−/−; P<0.0001), DOCA-salt treatment (P<0.0001), and fenofibrate treatment (P<0.0001). Subsequently, differences among groups were assessed by ANOVA followed by Turkey Honestly Significant Difference test (C). Likewise, multiple regression analysis indicated that tubulointerstitial fibrosis was determined by strain (WT vs Pex11a−/−; P<0.0001), DOCA-salt treatment (P<0.0001), and fenofibrate treatment (P<0.0001). Subsequently, differences among groups were assessed by ANOVA followed by Turkey Honestly Significant Difference test (D). In BSA-overload model, multiple regression analysis indicated that tubulointerstitial damage was significantly determined (P<0.0001) by strains (P=0.0117) and BSA treatment (P<0.0001). Subsequently differences among groups were assessed by ANOVA followed by Turkey Honestly Significant Difference test (E). Likewise, multiple regression analysis indicated that tubulointerstitial fibrosis was significantly determined (P=0.0001) by strains (P=0.0158) and BSA treatment (P<0.0001). Subsequently differences among groups were assessed by ANOVA followed by Turkey Honestly Significant Difference test (F). **P<0.01 by ANOVA followed by Tukey Honestly Significant Difference test. Scale bars, 50 μm. Error bars, mean±SD. WT indicates wild-type.
and division of pre-existing peroxisomes that depends on Pex11a.

Treatment with DOCA-salt decreased the abundance of peroxisomes and dysfunctional peroxisomes, which might indicate that pexophagy is induced to maintain functional peroxisomes. It is likely that impaired peroxisome elongation (a shift to be round) may drive pexophagy, as with mitophagy. However, the physiological and pharmacological regulation of the number of peroxisomes seems to be complex. The mechanism by which peroxisome biogenesis via budding from ER and pexophagy affects the abundance of peroxisomes in a DOCA-salt hypertension model needs to be clarified by additional studies.

Peroxisomes are intracellular organelles that are involved in non-ATP-generating fatty acid β oxidation and the control of ROS. A decrease in the number of peroxisomes results in an increase in lipid accumulation (Figures S1 and S4), which is always accompanied by an increase in ROS production. Pex11a deficiency-impaired antioxidant capacity (higher proportion of empty peroxisomes that contain only PMP70 and not catalase) leads to an intraperoxisomal redox disequilibrium. This metabolic–oxidative mismatch causes the further aggravation of peroxisomal functions and peroxisomal burnout, with consequent enhanced oxidative stress. 8-Iso-prostane levels and dihydroethidium staining might support this speculation. The excessive production of peroxisomal ROS as a result of fatty acid metabolism may overwhelm the cellular antioxidant defenses and cause significant damage to the cell structure or even cell death.

Figure 5. Pex11a deficiency decreases functional peroxisomes and aggravates oxidative stress. A, Representative immunofluorescence images show cortical sections of kidney stained with catalase (red), a peroxisomal matrix protein, and peroxisome membrane protein 70 (PMP70, green). The number of peroxisomes in the cortical areas (100,000 μm²) in mice under basal, deoxycorticosterone acetate (DOCA)-salt, and DOCA-salt and 0.05% fenofibrate (DOCA-Feno) conditions was counted. These experiments were performed independently from those described in Figure 1. B, The proportion of empty peroxisomes was calculated as follows: (detectable peroxisomes stained with PMP70−detectable peroxisomes stained with catalase)/detectable peroxisomes stained with PMP70. Multiple regression analysis indicated that proportion of empty peroxisomes was significantly determined (P<0.0001) by strains (P<0.0001), DOCA-salt treatment (P=0.0016), and fenofibrate treatment (P<0.0001). Subsequently, differences among groups were assessed by ANOVA followed by Turkey’s Honestly Significant Difference (HSD) test. C, The number of functional peroxisomes, as defined by the presence of both PMP70 and catalase. Multiple regression analysis indicated that the number of functional peroxisomes was significantly determined (P<0.0001) by strains (P<0.0001) and DOCA-salt treatment (P=0.0047) but not by fenofibrate treatment (P=0.1489). Subsequently, differences among groups were assessed by ANOVA followed by Turkey HSD test. D, Twenty-four-hour urinary 8-iso-prostane was measured. Multiple regression analysis indicated that urinary 8-iso-prostane was significantly determined (P<0.0001) by strains (P=0.0068), DOCA-salt treatment (P<0.0001), and fenofibrate treatment (P=0.0002). Subsequently, differences among groups were assessed by ANOVA followed by Turkey HSD test. *P<0.05, **P<0.01 by ANOVA followed by Tukey HSD test. Scale bar, 10 μm. Error bars, mean±SD. WT indicates wild-type.
Figure 6. Schematic diagram summarizing how Pex11a deficiency decreases functional peroxisomes and aggravates tubulointerstitial lesions through lipotoxicity. Albuminuria (induced by deoxycorticosterone acetate–salt hypertension and fatty acid overload models) results in the overloading of fatty acids to proximal tubule cells (PTC). Pex11a deficiency impairs peroxisome elongation and results in a reduction of functional peroxisomes, and then leads to an increase in lipid accumulation in PTC. Increased lipid accumulation accelerates the production of reactive oxygen species (ROS), which also results from the reduction of peroxisomes stained with catalase (impaired peroxisomal antioxidant defenses), and then induces tubulointerstitial injury and fibrosis and macrophage infiltration, and finally aggravates hypertension and albuminuria. Fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, is a strong inducer of Pex11a expression and improves peroxisome elongation and the abundance of functional peroxisomes and eventually ameliorates tubulointerstitial insult and albuminuria.

as an important mediator of lipotoxicity.32 These results suggest that decreased numbers of functional peroxisomes result in the aggravation of tubulointerstitial lesions induced by DOCA-salt hypertension, possibly through lipotoxicity. After FFA-bound albumin overloading, no significant difference in blood pressure was observed between WT mice and Pex11a−/− mice, but tubulointerstitial lesions of Pex11a−/− mice were significantly greater than those in WT mice (Figure 4). These results indicated that more severe tubulointerstitial lesions in Pex11a−/− mice were independent of increased blood pressure.

We first noticed Pex11a as a candidate gene for hypertension through an F2 analysis from Dahl salt-sensitive (Dahl S) and Lewis rats.35 The possibility that Pex11a might be one of the genes that contributes to Dahl S rats is described in the online-only Data Supplement (Pex11a as one of the candidate genes for Dahl salt-sensitive hypertension).

Although fenofibrate attenuated hypertension and tubulointerstitial lesions in both WT mice and Pex11a−/− mice, the latter still showed significantly higher levels of tubulointerstitial lesions. This observation suggests that the therapeutic effects of fenofibrate were attenuated in Pex11a−/− mice, and the induction of Pex11a contributes to the therapeutic effects of fenofibrate. As described above, fenofibrate might also affect peroxisome biogenesis from ER or pexophagy, which might be another therapeutic effect of fenofibrate that is observed equally in WT and Pex11a−/− mice. These aspects should be investigated further to establish therapeutic options to prevent the progression of CKD.

Perspectives

The present study illustrates an association between peroxisome biogenesis and tubulointerstitial lesions in CKD. Pharmacological or other maneuvers to activate the peroxisome system might be an excellent therapeutic strategy against hypertensive CKD.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**
- Pex11a-deficiency–impaired the abundance of functional peroxisomes in proximal tubule cells.
- Pex11a deficiency aggravated tubulointerstitial damage.
- Pex11a deficiency aggravated hypertension.

**What Is Relevant?**
- Peroxisomes play an important role in the pathological process of chronic kidney disease.

**Summary**

Pex11a-deficiency–impaired peroxisome biogenesis in proximal tubule cells and aggravated hypertension and tubulointerstitial damage through lipid toxicity.
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Methods

Experimental animals

Pex11a<sup>+</sup> mice were generated as described previously. Heterozygous mice were intercrossed to produce homozygous Pex11a<sup>-</sup> mice. These homozygous Pex11a<sup>-</sup> mice were backcrossed with C57Bl mice [wild type (WT) mice obtained from SLC Japan] five times prior to the generation of Pex11a<sup>-</sup> mice. Their offspring (Pex11a<sup>-</sup> and WT mice) were bred in the same temperature-controlled pathogen-free room with light from 0700 to 1900 (daytime) and used in the present experiment. The experimental protocols were approved by the National Cerebral and Cardiovascular Center Committee for Laboratory Animals, and all animal treatment was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were conducted so that the animals experienced minimal pain and discomfort.

DOCA-salt hypertension model

Eight-week-old mice were anesthetized with pentobarbital (25 mg/kg) and the left kidney was removed. After 10 days of recovery, mice were fed an 8% NaCl diet or the same diet supplemented with fenofibrate [a peroxisome proliferator-activated receptor alpha (PPARα) agonist; 0.05% wt/wt]. A slow-release (21-day) 75mg DOCA pellet (Innovative Research of America; Sarasota, FL) was implanted subcutaneously on the date the high-salt diet started, and the treatment was continued for 8 days. In addition, mice were housed in metabolic cages for 24-hr urine collection before (on day -2) and on days 2, 3, 6, and 8 of treatment with DOCA-salt.

Blood pressure was measured by telemetry in a separate set of DOCA-salt-treated Pex11a<sup>-</sup> and WT mice. Telemetry system transmitters (Data Sciences International; St. Paul, MN) were implanted as previously reported. Both WT and Pex11a<sup>-</sup> mice were anesthetized with pentobarbital (25 mg/kg), and a transmitter catheter was inserted into the left carotid artery, with the transmitter body placed subcutaneously in the lower right side of the abdomen, at the time of hemi-nephrectomy. After 10 days of recovery, a DOCA pellet was implanted and the high-salt diet was started. Systolic blood pressure (SBP) was measured with the telemetry system according to the manufacturer’s instructions before and throughout the DOCA-salt treatment period.

Free fatty acid (FFA)-bound bovine serum albumin (BSA)-induced renal tubular injury model

Mice were given consecutive daily intraperitoneal bolus injections of either 2.5 mg/g body weight FFA-bound BSA (Sigma-Aldrich, St Louis, MO) diluted in sterile PBS or an equal volume of sterile PBS as a control for 8 days. At day 8, 24-hr urine samples were collected.
Urinary analyses
Urinary concentrations of albumin, N-acetyl-β-D-glucosaminidase (NAG), and 8-iso-prostane were determined using an albumin enzyme-linked immunosorbent assay kit (Exocell; Shibayagi, Gunma, Japan), a Shiorogi NAG test (Shinogi & CO., LTD; Osaka, Japan), and an OxiSelect™ 8-iso-prostaglandin F2α ELISA kit (Cell Biolabs, Inc. CA), respectively. For all assays, samples were run in duplicate and the results were averaged.

Renal morphology and immunohistochemical analyses
Tubulointerstitial lesions and fibrosis were evaluated in renal tissue sections (5 μm) with Masson Trichome staining. Ten fields from the cortical areas were selected randomly per mouse. Tubulointerstitial lesions were defined as the accumulation of tubular dilatation/atrophy, tubule vacuole and cast formation, interstitial edema, and epithelial cell necrosis. Tubulointerstitial fibrosis was defined as the accumulation of extracellular matrix (stained blue). The extent of tubulointerstitial injury and fibrosis was defined as a ratio relative to the entire cortical area with Image J software (National Institutes of Health, Bethesda, MD). Oil Red O staining was performed as described previously. Immunohistochemical staining was performed as described previously. Desectioned kidney sections were heated for 20 min at 121 °C in 10 mM citric acid solution for antigen retrieval and then incubated with antibody against F4/80 (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was detected using the Histofine Simple Stain MAX-PO (mouse) kit (Nichirei, Tokyo, Japan) and peroxidase stain DAB kit (Nacalai Tesque, Kyoto, Japan). The nuclei were stained with hematoxylin.

For proximal tubule peroxisome staining, immunofluorescence staining was performed as described previously. The sections were incubated with either rabbit antibody against peroxisome membrane protein 70 (anti-PMP70, Invitrogen), goat anti-catalase (Santa Cruz Biotechnology, Santa Cruz, CA), or goat anti-megalin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies overnight at 4 °C. The primary antibodies were detected with Texas Red-conjugated donkey anti-goat secondary antibodies (Abcam, Cambridge, UK) and Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibodies (Abcam, Cambridge, UK). Images were acquired using a confocal laser-scanning microscope (Olympus, Tokyo, Japan) and analyzed using Image J software. Ten randomly selected independent fields (100,000 μm²) were photographed per mouse. Peroxisomes were counted using the Particle Analysis package of Image J. The proportion of empty peroxisomes was calculated as follows: (detectable peroxisomes stained with PMP70 – detectable peroxisomes stained with catalase)/detectable peroxisomes stained with PMP70.

Immunoelectron microscopy
Immunoelectron microscopic staining was performed as described previously.
sections (80 nm) were incubated with rabbit anti-PMP70 antibodies (Invitrogen) and visualized using gold particles (Anti Rabbit IgG; British BioCell International, Cardiff, UK). To quantitatively evaluate peroxisome morphology, 10-20 fields were examined, and the roundness of 200 peroxisomes was measured per mouse using the Measure Analysis package of Image J based on the following equation: roundness = perimeter^2/(4×π×area). Roundness was categorized according to the following parameters (for visual purpose only) and is presented in Figure 2C and Supplemental Figure 3C and 3D: I < 1.05, 1.05 ≤ II < 1.08, 1.08 ≤ III < 1.11, 1.11 ≤ IV < 1.14, 1.14 ≤ V < 1.17, 1.17 ≤ VI < 1.2, 1.2 ≤ VII < 1.25, and 1.25 ≤ VIII. Statistical analyses (Wilcoxon/Kruskal-Wallis test described below) were performed using raw numerical values rather than the categorical values described above.

**Dihydroethidium (DHE) staining**

Frozen sections were incubated with a fluorophore that is sensitive to O_2^-·. Dihydroethidium (DHE, 10 mol/L, Sigma) specifically reacts with intracellular O_2^-· and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining. 

**Transcriptome analysis**

RNA was extracted from kidneys using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Messenger RNA (mRNA) expression levels were determined by real-time PCR using a commercial kit (Applied Biosystems, Foster City, CA). All samples were analyzed in duplicate with a dual assay that measured both the gene of interest and β-actin as an internal control, as described previously.

**Quantification and statistical analysis**

The peroxisome roundness was analyzed by the Wilcoxon/Kruskal-Wallis test and other data were analyzed using a one-way analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), or multiple regression analysis, and then Tukey’s HSD test or Bonferroni correction using the JMP statistical analysis package (SAS Institute, Cary, NC). P < 0.05 was considered significant. Data are presented as means ± SD.
**PexIIa as one of the candidate genes for Dahl salt-sensitive hypertension**

Our previous F2 analysis indicated three possible candidate chromosomal regions for hypertension: Ch1 (possibly 2 loci, one for around Klk1 and the other around Nirk3), Ch10, and Ch12. We determined the expression levels of 36 transcripts in the candidate Ch1 region, and PexIIa and Nmb were concluded to be the candidate genes in Ch1 (Supplemental Figure S9).

**Supplemental Figure S9. PexIIa might be a good candidate for susceptible genes for salt-sensitive hypertension.** Dahl salt-sensitive and Lewis rats were purchased from SLC Japan (Shizuoka, Japan). At four weeks of age, rats began to be fed with either a high-salt diet (8%) or normal-salt diet (0.32%) for 8 weeks. (A) Transcriptome analyses of 36 genes, which resided in the Ch1q31 region of QTL for blood pressure, were performed by real-time RT PCR. (B) In the first step, expression levels of these transcripts were assessed in various tissues of Dahl and Lewis rats, including thymus, spleen, pineal gland, pituitary gland, brain stem, cerebellum, cerebrum, aorta, adrenal gland, kidney, liver, and heart, under basal conditions with a normal-salt diet (n = 1).
(C) In several tissues including the heart, kidney, adrenal gland, aorta, and pineal gland, expression levels were assessed in Dahl and Lewis rats under either high-salt diet or normal-salt diet feeding (n = 1). (D) Based on this screening, expression levels of several transcripts including *Pex11a, Isg20, Mfge8, Anpep, Nmb*, and *Sle28a1* were re-assessed in the kidneys of Dahl and Lewis rats either under high-salt diet or normal-salt diet feeding. Expression levels of *Pex11a* and *Nmb* appeared to be due to the strain difference, and these might be good candidates for susceptible genes for salt-sensitive hypertension. *P < 0.05* by ANOVA followed by Tukey’s HSD test.
The expression levels of *Pex11a* in Dahl S rats were lower than those in Lewis rats under both normal and high-salt diet feeding. The numbers of peroxisomes in Dahl S rats were lower than those of Lewis rats under high-salt diet feeding (Supplemental Figure S10).

Moreover, fenofibrate attenuated the increase in blood pressure in Dahl S rats under high-salt diet feeding, as reported by other investigators\(^{10,11}\) and induced the expression of *Pex11a* but not *Nmb* (data not shown). Thus, it is likely that *Pex11a* rather than *Nmb* contributes to salt-sensitive hypertension in Dahl S rats.

**Supplemental References**


Figure S1

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Supplemental Figure S1. Pex11a deficiency aggravates lipid accumulation in proximal tubules in DOCA-salt hypertension mice.

Representative images of cortical sections from basal, DOCA-salt (DOCA)-treated, and combined DOCA-salt and 0.05% fenofibrate (DOCA-Feno)-treated (8 days) mice. Renal lipids were detected with Oil Red O staining. Numerous small lipid droplets were observed in proximal tubules after treatment with DOCA-salt, and were more obvious in Pex11a−/− mice than in WT mice. Scale bar: 50 μm.
Figure S2

A  Basal  DOCA  DOCA-Feno

WT

Pex11α−/−
Figure S2

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Figure S2

**P < 0.0001 by ANOVA**

1. Basal
2. DOCA
3. DOCA-Feno

**P < 0.0001 by ANOVA**

1. Basal
2. BSA

**P < 0.01 by ANOVA**

1. Basal
2. BSA
Supplemental Figure S2. Pex11a deficiency aggravates macrophage infiltration induced by DOCA-salt treated and free fatty acid (FFA)-bound albumin overload. (A and B) Representative images of F4/80 immunohistochemical analysis in the renal cortex of the mice under basal, DOCA-salt (DOCA)- or DOCA-salt, 0.05% fenofibrate (DOCA-Feno)-treated and FFA-bound albumin (bovine serum albumin, BSA) overload conditions. Macrophage (arrows) infiltration was more prominent in the tubulointerstitial region in the cortex of Pex11a−/− mice than WT mice. (C and D) The number of F4/80-positive cells in 10 high-power fields (B, hpf) was counted. In DOCA-salt hypertensive model, multiple regression analysis indicated that the number of macrophages was significantly determined (P < 0.0001) by strains (P < 0.0001), DOCA-salt treatment (P < 0.0001), and fenofibrate treatment (P < 0.0001). Subsequently, difference among groups was assessed by ANOVA followed by Turkey’s HSD test (C). In BSA overload mode, multiple regression analysis indicated that the number of macrophages was significantly determined (P = 0.0001) by strains (P = 0.0158) and BSA treatment (P = 0.0001). Subsequently, differences among groups were assessed by ANOVA followed by Turkey’s HSD test (D). (E) Assessment of urinary N-acetyl-β-D-glucosaminidase (NAG) excretion. 24-hr urine was collected to assess the NAG excretion per day. *P < 0.05. **P < 0.01
Figure S3

![Graph showing Systolic Blood Pressure (mmHg) over Days on BSA treatment. The graph compares WT (2) with Pex11a-/- (2).]
Supplemental Figure S3. Systolic blood pressure (SBP) in free fatty acid (FFA)-bound albumin-overload mice

WT mice and Pex11a−/− mice were treated with FFA-bound albumin (2.5 mg/g bovine serum albumin, BSA) for 10 days. SBP was monitored by telemetry. BSA-overload increased blood pressure in the early phase, but blood pressure returned to normal in the later phase. No significant difference in SBP (n = 2, mean value) was observed between WT mice and Pex11a−/− mice.
Figure S4

WT

Pex11a−/−
Supplemental Figure S4. Pex11a deficiency aggravates lipid accumulation in proximal tubules in free fatty acid (FFA)-bound albumin-overload mice.
Representative images of cortical sections from basal and FFA-bound albumin (2.5 mg/g bovine serum albumin, BSA, 8 days)-treated mice. Renal lipids were detected with Oil Red O staining. Numerous small lipid droplets were observed in proximal tubules after treatment with BSA, and were more obvious in Pex11a<sup>−/−</sup> mice than in WT mice. Scale bar: 50 μm.
Figure S5

A Basal

PTMP70
Megalnin

WT

Pex11a−/

BSA
Figure S5

$P < 0.0001$ by ANOVA
Supplemental Figure S5. Peroxisome abundance in the kidneys of free fatty acid (FFA)-bound albumin-overload mice

(A) Representative immunofluorescence images with staining for megalin (red), a protein that is highly expressed in proximal tubule cells, which was used to localize proximal tubules, and peroxisome membrane protein 70 (PMP70; green), which was used to represent peroxisomes. *Pex11a−/−* mice had fewer peroxisomes than WT mice under both basal and bovine serum albumin-treated (BSA, 8 days) conditions. (B) The number of peroxisomes in proximal tubules of the cortical areas (100,000 μm²) in each mouse was counted with Image J software in WT and *Pex11a−/−* mice. Multiple regression analysis indicated that the number of peroxisomes was significantly determined by strains (*P* < 0.0001) and BSA treatment (*P* < 0.0001). Subsequently differences among groups were assessed by ANOVA followed by Turkey’s HSD test. *P* < 0.05, **P* < 0.01 Scale bar: 10 μm.
Figure S6

BSA-WT

BSA-Pex11a/-
**Figure S6**

**C**

- **Basal**
  - WT: Functional peroxisomes/5,000 μm²
  - Pex11a-/: Functional peroxisomes/5,000 μm²

- **BSA**
  - WT: Functional peroxisomes/5,000 μm²
  - Pex11a-/: Functional peroxisomes/5,000 μm²

**D**

- **Basal**
  - WT: Proportion of empty peroxisomes
  - Pex11a-/: Proportion of empty peroxisomes

- **BSA**
  - WT: Proportion of empty peroxisomes
  - Pex11a-/: Proportion of empty peroxisomes
**Supplemental Figure S6.** Pex11a deficiency decreases functional peroxisomes and aggravates oxidative stress in free fatty acid (FFA)-bound albumin-overload model. 

**(A and B)** Representative immunofluorescence images show cortical sections of kidney stained with catalase (red), a peroxisomal matrix protein, and peroxisome membrane protein 70 (PMP70, green). The number of peroxisomes in the cortical areas (100,000 μm²) in mice under FFA-bound albumin (bovine serum albumin, BSA) overload condition was counted. **(C)** The number of functional peroxisomes, as defined by the presence of both PMP70 and catalase. Multiple regression analysis indicated that the number of functional peroxisomes was significantly determined \((P < 0.0001)\) by strains \((P < 0.0001)\) and BSA treatment \((P = 0.0642)\). Subsequently, differences among groups were assessed by ANOVA followed by Turkey’s HSD test. **(D)** The proportion of empty peroxisomes was calculated as follows: (detectable peroxisomes stained with PMP70 - detectable peroxisomes stained with catalase)/detectable peroxisomes stained with PMP70. Multiple regression analysis indicated that the number of functional peroxisomes was significantly determined \((P < 0.0001)\) by strains \((P < 0.0001)\) and BSA treatment \((P = 0.0247)\). Subsequently, differences among groups were assessed by ANOVA followed by Turkey’s HSD test. \(*P < 0.05\), \(**P < 0.01\.** Scale bars: 10 μm.
Figure S7

A  Basal  DOCA  DOCA-Feno

WT

Pex11α−/−  PEG-SOD
Figure S7

$P < 0.001$ by ANOVA

Fluorescence intensity per field

- Basal
- DOCA
- DOCA-Feno

WT
Pex11a−/−
Supplemental Figure S7. Pex11a deficiency aggravates oxidative stress.

(A) Representative images of cortical sections of kidney in WT and Pex11a<sup>−/−</sup> mice. The increased fluorescent signal in DOCA-salt-treated Pex11a<sup>−/−</sup> mice was almost completely abolished by coincubation with 500 U/mL polyethylene glycol-superoxide dismutase (PEG-SOD), which represents specific evidence for the presence of increased reactive oxygen species. There was an increase in fluorescence signal in cortical sections of WT and Pex11a<sup>−/−</sup> mice after DOCA-salt (DOCA) treatment for 8 days, but this was restored by treatment with 0.05% fenofibrate (Feno). Images were acquired using a confocal laser-scanning microscope. (B) The fluorescence signal intensity in the cortical areas (10 fields) in each mouse in A was quantified using the Measure Analysis package of Image J in 6 groups of mice. Multiple regression analysis indicated that the fluorescence signal intensity was significantly determined ($P < 0.0001$) by strains ($P < 0.0001$), DOCA-salt treatment ($P < 0.0001$), and fenofibrate treatment ($P = 0.0028$). Subsequently, differences among groups were assessed by ANOVA followed by Turkey’s HSD test. *$P < 0.05$. Scale bar: 50 μm.
Figure S8

A  

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Bar scale = 500 μm
Figure S8

**B**

$P < 0.0001$ by Kruskal-Wallis test

**C**

$P < 0.0015$

**D**

$P < 0.0015$
Supplemental Figure S8. Ultramicroscopic structural analysis of peroxisome morphology in DOCA-salt mice

(A) Representative immunoelectron microscopy images with staining for PMP70. Tubular or elongated peroxisomes are visible (arrows). MT: mitochondria. Peroxisome morphology in (A) was measured using the Measure Analysis package of Image J based on the following equation: roundness = perimeter$^2$(4$\pi$area). Roundness is shown in a scatter diagram (B) and categorized (C and D) according to the following parameters: I < 1.05, 1.05 ≤ II < 1.08, 1.08 ≤ III < 1.11, 1.11 ≤ IV < 1.14, 1.14 ≤ V < 1.17, 1.17 ≤ VI < 1.2, 1.2 ≤ VII < 1.25, and 1.25 ≤ VIII. Peroxisome became more round in WT mice but not in Pex11a$^{-/-}$ mice after DOCA-salt treatment, and this was restored by 0.05% fenofibrate treatment. The peroxisome roundness was analyzed by the Wilcoxon/Kruskal-Wallis test followed by the Bonferroni correction using the JMP statistical analysis package, *P < 0.0015. The abbreviations of BW, BP, DW, DP, DFW, and DFP represent Basal WT, Basal Pex11a$^{-/-}$, DOCA-salt (DOCA)-treated WT, DOCA-treated Pex11a$^{-/-}$, DOCA and fenofibrate (DOCA-Feno)-treated WT, and DOCA-Feno-treated Pex11a$^{-/-}$, respectively. Scale bar: 1μm.