Video Article

Induction of Nephrotic Syndrome in Mice by Retrobulbar Injection of Doxorubicin and Prevention of Volume Retention by Sustained Release Aprotinin

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Abstract

Nephrotic syndrome is the most extreme manifestation of proteinuric kidney disease and characterized by heavy proteinuria, hypoalbuminemia, and edema due to sodium retention and hyperlipidemia. To study the pathophysiology of this syndrome, rodent models have been developed based on the injection of toxic substances such as doxorubicin causing podocyte damage. In mice, only few strains are susceptible to this model. In wildtype 129S1/SvImJ mice, the administration of doxorubicin by rapid intravenous injection to the retrobulbar sinus induces experimental nephrotic syndrome that features all the symptoms of human disease including sodium retention and edema. After the onset of proteinuria, mice exhibit increased urinary serine protease activity that leads to the activation of the epithelial sodium channel (ENaC) and sodium retention. Pharmacological inhibition of urinary serine proteases by the treatment with sustained release aprotinin abrogates ENaC activation and prevents sodium retention. This model is ideal to study the pathophysiology of proteasuria, *i.e.*, the excretion of active serine proteases that cause ENaC activation by the proteolysis of its γ-subunit. This can be regarded as the primary mechanism of ENaC activation and sodium retention in proteinuric kidney disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/57642/

Introduction

Nephrotic syndrome is characterized by heavy proteinuria, hypoalbuminemia, edema and hyperlipidemia, and can be regarded as the most extreme manifestation of proteinuric kidney disease. In rodents, experimental nephrotic syndrome can be induced by single injection of anthracyclines or puromycin which leads to podocyte damage and resembles human minimal change disease and focal segmental glomerulosclerosis (FSGS)[1]. After its first description in 1955 by Frenk *et al.*[2], puromycin nucleoside nephrosis (PAN) in rats has become a standard model to investigate the pathophysiology of nephrotic syndrome in numerous studies[3,4,5,6]. In mice, the corresponding model can be induced by the anthracycline doxorubicin[7]. However, there is a strong strain-dependency that is genetically determined by at least two genetic loci[8]. In addition, there are differences in the proteinuric response and course of the nephrotic syndrome[9,10]. Using 129S1/SvImJ mice and rapid intravenous injection of doxorubicin via the retrobulbar sinus, proteinuria responses reach values that are sufficient to induce the typical features of nephrotic syndrome, particularly volume retention characterized by ascites and nearly sodium-free urine[7]. Sodium retention in experimental nephrotic syndrome has been presumed to be the result of activation of the epithelial sodium channel (ENaC) in the distal tubule by aberrantly filtered serine proteases such as plasmin causing proteolysis of its γ-subunit[4,11,12]. Recently, this concept was proven in nephrotic mice which were protected from proteolytic ENaC activation and sodium retention by treatment with the serine protease inhibitor aprotinin that was equally effective as the ENaC blocker amiloride[13]. To ensure continuous delivery to the distal tubule, aprotinin was administered via subcutaneously implanted sustained release pellets. Future studies are required to identify the serine proteases that are responsible for proteolytic ENaC activation in nephrotic syndrome which is thought to parallel the human situation. For this purpose, doxorubicin-induced nephrotic syndrome is a valuable model that can be used in wild-type mice or expanded to genetically engineered mice. Its advantages include low cost of the drug, lower complexity of management and good reproducibility[14].

In this article, we demonstrate the induction of experimental nephrotic syndrome by rapid intravenous injection of doxorubicin to the retrobulbar sinus and implantation of sustained-release pellets containing aprotinin to inhibit urinary serine protease activity as measured with a chromogenic assay.

Protocol

All methods were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German law for the welfare of animals, and they were approved by local authorities (Regierungspräsidium Tübingen).

1. Induction of Experimental Nephrotic Syndrome by Doxorubicin Injection to the Retrobulbar Sinus

1. Prepare a 0.5 mL syringe with a mounted 30G cannula by marking the stop position of the piston.
2. Calculate injection volume of doxorubicin for male (7.25 µL/g body weight (bw) equal to 14.5 µg/g bw doxorubicin) and female mice (6.9 µL/g bw equal to 13.8 µg/g bw doxorubicin) according to the body weight from the morning.
   NOTE: The given dose is appropriate for induction of nephrotic syndrome. Minor changes in dosage lead to a crucial different course of nephropathy reaching from mild chronic kidney disease with only small changes in glomerular filtration rate (12.6 µg/g bw doxorubicin) to acute kidney failure (15.4 µg/g bw doxorubicin)[7,15].
3. Warm the calculated amount of doxorubicin solution (2 µg/µL) in a 37 °C warm chamber.
   Caution: Doxorubicin is harmful if swallowed and can cause cancer. Always wear gloves if working with doxorubicin and avoid any skin contact.
4. Prefill the syringe with doxorubicin solution until the marking point and set the balance to zero. Fill the syringe with the injection volume and check the volume by weighing the syringe.
5. Narcotize the mouse deeply with 5 vol% isoflurane using a vaporizer and oxygen flow of 2 L/min.
   1. Do not use ointment on the eyes prior to the injection. The operator needs a clear view of the orbital cavity for performing a safe and successful retrobulbar injection.
   2. Assess the level of anesthesia by pedal reflex and adjust anesthetic delivery if necessary before starting injection.
6. Place the mouse at a right lateral recumbency with its back facing to the operator's body and its head facing to the operator's injection hand.
   NOTE: The procedure is described for a right-handed person, for left-handed persons performing the injection with the left hand it is easier to do the position and injection reversed right to left.
7. Carefully protrude the mouse's left eyeball from the eye socket by applying gentle pressure to the skin, dorsal and ventral to the eye.
8. Puncture the left retrobulbar sinus from the inner eye angle (medial palpebral commissure). Avoid any contact with the mouse´s eyeball.
9. Slightly tilt the syringe and inject entire volume in one go. Make sure that the volume is injected without any resistance and without any signs of extravasation such as exopthalmus or leakage from the injection site.
10. Since doxorubicin is a highly toxic substance, check well-being at least once a day by using a score-sheet according to Morton and Griffiths *et al.*[16] and pay special attention to the injection site. If any signs of extravasation like exopthalmus, impaired eyelid closure or any signs of necrosis like swelling or skin lesions occure after injection or in the following days, the mouse should be euthanized.

2. Implantation of Sustained-Release Pellets Containing Aprotinin

1. Order pellets with the desired dose per day and release duration in advance. In case of aprotinin, choose a dose of 1 mg per day to be released over 10 days.
   NOTE: In this study, the aprotinin pellets contain a dose of 10 mg.
   1. Store the pellets under dry conditions and avoid any exposure to humidity.
2. Prepare required items for the surgery, including a pair of hair scissors, a pair of skin preparation scissors, a scalpel, surgical tweezers, two pairs of tissue tweezers, a needle holder, and 15 cm monofile suture non resorbable.
   1. Sterilize the instruments using a heat sterilizer for 5 min at 240 °C. Before using the instruments, wait for 5 min until they reach room temperature.
      1. Avoid any contact of sterilized instrument tips to nonsterile surfaces.
      2. Avoid skin burn by hot instruments.
3. Narcotize mouse with isoflurane 5 vol% followed by 1.5 vol% using a vaporizer and oxygen flow of 2 L/min.
4. Place the mouse in a prone position on a warming device covered with a layer of gauze to avoid thermal injuries to the mouse. Surface temperature is about 37 °C.
5. Protect the eyes with ointment.
6. Remove the hair on the middle back in an area of about 0.5 cm^2 using scissors or a hair trimmer and disinfect the hairless skin with a disinfectant suitable for skin disinfection. Remove as less hair as possible so it stays more difficult for the mouse to reach the wound and thread ends later and to keep body temperature.
7. Incise the hairless skin with a scalpel in cranio-caudal direction in a length of about 5 mm. Prepare a left lateral pouch of about 1 cm depth in the subcutaneous connective tissue using a blunt preparation.
   1. Assess the level of anesthesia by pedal reflex and adjust anesthetic delivery if necessary before starting surgery.
8. Insert one 10-day release pellet into the prepared left lateral pouch using sterile tissue tweezers. Leave the pellet at the bottom of the pouch in a planar position.
   1. Avoid any contact of the pellet to fluid and humidity till placed in the prepared pouch.
9. Close the skin with 2-3 sutures. Only leave very short thread ends to make it more difficult for the mouse to open the sutures by gnawing.

10. Place the mice individually to reduce postoperative distress and to prevent opening of the sutures by gnawing. Keep the mouse in view until it has regained sufficient consciousness after narcosis.
    NOTE: There is no need for postoperative pain management since this intervention is well tolerated without any signs of discomfort or pain. Alternatively we recommend topical analgesics, e.g. a drop of bupivacaine on the incision prior to closure which would provide up to 8 h of analgesia.

3. Assessment for Model Induction, Signs of Nephrotic Syndrome and well-being

1. Collect morning urine (08:00 am) into a reaction cup (1.5 mL) every day from the injection day by massaging the bladder.
    1. Measure proteinuria by using Bradford protein assay and normalize to creatinine.
       NOTE: For induction of nephrotic syndrome, proteinuria should reach a threshold of 120 mg/mg creatinine between day 7 and 10 after doxorubicin injection.
2. Look for the development of ascites. Check for increase of abdominal circumference or overhanging flanks.
3. Weigh the mouse, the food pellets and the drinking bottle in the morning (08:00 am) every day.
4. Check well-being at least once a day by using a score-sheet according to Morton and Griffiths *et al.*[16]

4. Measurement of Urinary Serine Protease with a Chromogenic Assay

1. Prepare substrate working solution by adding 15 mL of sterile phosphate buffered saline (PBS) to a bottle of 25 mg of lyophilised chromogenic substrate S-2251, yielding a concentration of 2 mM. Dissolve aprotinin in PBS to obtain an aprotinin solution with a concentration of 2 mg/mL.
    1. Store prepared solutions at -20 °C. This significantly slows the auto-degradation of S-2251. Do not use old solutions with a strong shade of yellow.
2. Use freshly prepared or thawed substrate working solution and warm it in a 37 °C heat chamber.
3. Thaw urine samples at room temperature.
    1. Prevent protease degradation by avoiding repeated thawing and freezing. Store urine samples at -20 °C.
4. Use a 96 microwell plate suitable for photometric measurement. Add 3 µL of undiluted urine in each of two wells and add 50 µL of substrate working solution with 3 µL of either PBS or aprotinin solution to the wells. Cover the plate with a plate sealer.
5. Incubate the covered microwell plate for 60 min in a 37 °C heat chamber.
6. Measure the optical density of each well at 450 nm using a microplate reader.
7. Take the difference between the OD without aprotinin and with aprotinin as urinary aprotinin-sensitive serine protease activity.
   NOTE: Measurement of a paired sample with and without aprotinin increases specificity of the assay since the substrate could also be cleaved by other proteases that do not play a pathophysiological role.

Representative Results

After the induction of isoflurane narcosis, doxorubicin was rapidly injected via the left retrobulbar sinus. The entire volume of 7.25 µL/g bw was injected without any resistance and extravasation proving correct intravenous location of the cannula. The mouse recovered from narcosis rapidly and had no impairment on that day and thereafter. Particularly, there was no sign of damage at the left eye. After doxorubicin injection, food and fluid intake dropped over the first 3 days due to a general toxic effect of doxorubicin and recovered thereafter (**Figure 1a**). During the first 3 days after injection, the mouse lost 3.4 % of its body weight owing to inappetence (**Figure 1b**). Starting on the 5th day, the mouse developed marked proteinuria (**Figure 1c**) that was followed by a massive decline of urinary sodium excretion despite adequate food intake (**Figure 1a, b**). This sodium retention lead to a massive gain of body weight by 22.2 % from day 3 until day 10 and was accompanied by ascites. As a consequence of proteinuria, the mouse developed hypoalbuminemia (**Figure 1c**). In a plasma sample taken on day 10, hyperlipidemia was visible. These data show that doxorubicin given by the retrobulbar sinus rapidly and impressively induces a full-blown nephrotic syndrome in wildtype 129S1/SvImJ mice.

Sodium retention in experimental nephrotic syndrome was linked to the activation of ENaC by aberrant filtration of serine proteases such as plasmin[11,12]. If this concept holds true, then the inhibition of urinary serine proteases by aprotinin should protect from ENaC activation and sodium retention. To study the effect of aprotinin on ENaC activation, we treated a nephrotic mouse with aprotinin. As aprotinin is rapidly cleared by glomerular filtration, we chose drug administration using a sustained-release pellet to ensure continuous availability of aprotinin in the distal tubule over 10 days. On the third day after doxorubicin injection, one mouse was implanted with an aprotinin-containing pellet and a control mouse received a placebo pellet under isoflurane narcosis. Animals recovered rapidly form narcosis and the wound healed without any problems. From day 5 on, proteinuria developed in both mice to a similar extent (**Figure 2a**). However, only the placebo-treated nephrotic mice experienced sodium retention (**Figure 2b**) and body weight gain (**Figure 2c**) indicating ENaC activation. In contrast, the aprotinin-treated mouse was protected from sodium retention and body weight gain (**Figure 2b**, **c**). This clearly demonstrates that urinary serine protease activity is the cause of sodium retention in nephrotic syndrome.

The aprotinin concentration achieved in urine and plasma was measured with an ELISA. As shown in **Figure 3a**, urinary aprotinin concentration peaked soon after the implantation and was constant thereafter. Mean urinary aprotinin concentration was 207 ± 29 µg/mL (32 ± 4 µM) whereas plasma aprotinin concentration after 10 days of treatment was 13 µg/mL (2 µM), which is comparable to the plasma concentration achieved in aprotinin-treated patients[17]. The effect of aprotinin on urinary serine protease activity was measured with a chromogenic assay using urine samples taken during the course of experimental nephrotic syndrome. This assay is based on the hydrolysis of the peptide bond in the substrate releasing p-nitroaniline that can be easily detected by photometry at 450 nm. As shown in **Figure 3b**, serine protease activity increased rapidly in urine from the placebo-treated nephrotic mouse paralleling the onset of proteinuria. In contrast, urinary serine protease activity was completely inhibited in the aprotinin-treated nephrotic mouse coinciding with the prevention of ENaC activation and sodium retention shown in **Figure 2b, c**.

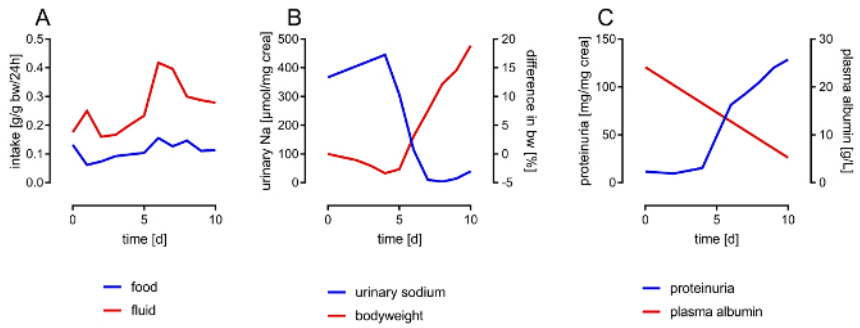

Figure 1: Course of food and fluid intake (a), urinary sodium excretion and body weight (b), and proteinuria and plasma albumin concentration (c) before and after the induction of experimental nephrotic syndrome. After a decline in food and fluid intake over the first three days after doxorubicin, mice show a nearly constant intake. After five days, the development of proteinuria starts and reaches nephrotic threshold between day 7 and 10, leading to full-blown nephrotic syndrome with edema formation, sodium retention and hypoalbuminemia. Please click here to view a larger version of this figure.

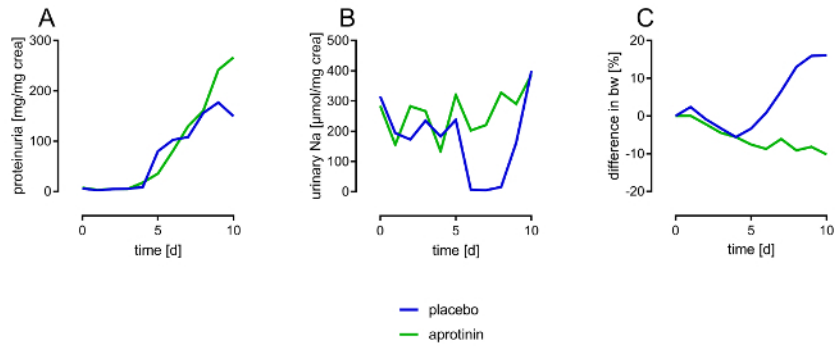

Figure 2: Course of proteinuria (a), urinary sodium excretion (b) and body weight (c) in a mouse treated each with aprotinin or placebo pellet on day 3 after doxorubicin injection. Despite the development of proteinuria in a similar extent aprotinin protects against sodium retention and edema formation caused by ENaC activation during nephrotic syndrome. Please click here to view a larger version of this figure.

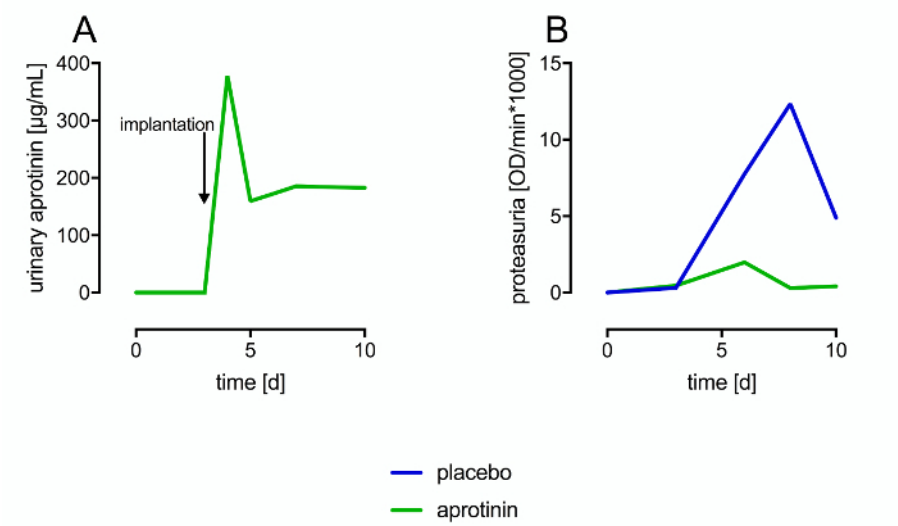

Figure 3: Course of urinary aprotinin concentration after the implantation of the pellet with plasma concentration of aprotinin. Course of urinary serine protease activity measured with the chromogenic assay described in step 4. Urinary aprotinin concentration peaks soon after the implantation and is constant thereafter. Serine protease activity increases rapidly in urine during nephrotic syndrome, paralleling the onset of proteinuria. Aprotinin *in vivo* prevents against increase in urinary serine protease activity. Please click here to view a larger version of this figure.

Discussion

Here, we demonstrate that doxorubicin injection via retrobulbar sinus injection induces experimental nephrotic syndrome in 129S1/SvImJ mice with proteinuria, sodium retention, hypoalbumenia and hyperlipidemia. However, there are two critical issues that have to be taken into account when using this model. Firstly, the model induction is strictly dose dependent and deviations of doxorubicin dose as small as 0.3 µg/g affect the response of the mice[15]. When injected with a lower dose such as 14 µg/g bw or below, doxorubicin causes only non-nephrotic proteinuria that is not associated with overt sodium retention, ascites and hyperlipidemia. In contrast, injection of a higher dose leads to acute renal failure and high acute mortality. According to the dose and response of the mice, doxorubicin causes a course resembling human acute kidney injury (AKI), chronic kidney disease with preserved glomerular filtration rate (CKD stage G1-3) or progressive chronic kidney disease with reduced glomerular filtration rate (CKD G4-5) leading to uremia[7,15].

Secondly, this model is limited to certain mouse strains and the widely used C57Bl/6 strain is resistant[8]. Furthermore, genetical contamination as they might be present during a backcross may lead to a significantly lower response rate. This can be avoided to some extent by increasing the doxorubicin dose. However, the dose of doxorubicin used for model induction (14.5 µg/g bw) is already close to the described LD50 between 15-17 µg/g bw in this strain[7,18].

The use of this mouse model opens up the possibility to study different highly innovative questions concerning the nephrotic syndrome, reaching from proteinuria and edema formation to endocrinological dysregulations and renal anemia[15]. In this sense, doxorubicin-induced nephropathy covers the wide spectrum of human CKD and is a good alternative to current models utilized in nephrology research such 5/6 nephrectomy[19,20] or unilateral ureter ligation[21]. Furthermore, it is possible to apply the model to genetically engineered mice to study the role of a certain gene of interest in the course of the model such as the lack of the serum-and-glucocorticoid kinase 1 (SGK1)[7] or many others.

A special feature of the presented model is the occurrence of proteasuria, *i.e.*, the excretion of active serine proteases that cause ENaC activation by proteolysis of its γ-subunit. This leads to excretion of a nearly sodium-free urine and volume retention characterized by rapid body weight gain and development of ascites after onset of proteasuria. However, this phenomenon is transient, and after 15 days, mice spontaneously loose ascites and weight although proteinuria persists. The reason, however, remains elusive. This paradoxical behavior was also observed in studies with nephrotic rats[4] and seems to represent a general feature of experimental nephrotic syndrome in rodents.

Urinary serine protease activity was measured with a chromogenic assay that is simple and fast. It is based on the hydrolysis of the amide bond of p-nitroaniline linked to a peptide of 4 amino acids length that can be cleaved by different proteases. Generally, chromogenic substrates are not specific for a certain protease and there is substantial overlap of the proteases that cleave a particular substrate. The here used substrate is a preferred substrate of trypsin-like serine proteases such as plasmin or plasma kallikrein[22]. To increase specificity, protease activity should always be determined from a paired sample with and without an inhibitor[23,24]. In this study, aprotinin was used to define activity of trypsin-like serine proteases. By calculating the aprotinin-sensitive activity from the difference, the activities of other proteases are cancelled out. In the nephrotic mouse treated with aprotinin *in vivo,* urinary serine protease activity was not increased and most importantly coincided with abrogation of sodium retention. Thus, urinary serine protease activity is not only causal for sodium retention in nephrotic syndrome, but can also be regarded a biomarker to predict efficacy of a serine protease inhibitor in nephrotic syndrome, which might become a new therapeutically approach in treatment of nephrotic syndrome. In the study by Bohnert *et al.*[13], the serine protease inhibitor camostat and plasminogen conversion inhibitor tranexamic acid were not effective in preventing sodium retention which coincided with a failure to normalize urinary serine protease activity.

Although doxorubicin is a highly toxic substance, retrobulbar injection is safe and not associated with any harm that impaired the well-being of the animal. One could argue that tail vein injection would be a more suitable approach reducing animal distress. In a comparative study, we found out that induction via tail vein injection of doxorubicin was inferior to retrobulbar route due to a higher rate of mice that did not develop

nephrotic syndrome (34% non-responder) compared to those induced by the retrobulbar route (0-15% non-responder). In addition, proteinuria by tail vein injection was lower leading to attenuated sodium retention. This might be explained by the differences in pharmacokinetics and a higher peak concentration of doxorubicin achieved after rapid retrobulbar injection that is essential for model induction by podocyte damage. However, it must be emphasized that retrobulbar injection needs a highly skilled and experienced operator to avoid complication related to extravasation of even minute amounts of doxorubicin.

In conclusion, experimental nephrotic syndrome by doxorubicin is a versatile model of proteinuric kidney disease in 129S1/SvImJ mice that allows to investigate highly current scientific questions, most importantly proteasuria.

Disclosures

The authors have nothing to disclose.

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