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Nephron Prorenin Receptor Deficiency Alters Renal Medullary Endothelin-1 and Endothelin Receptor Expression

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Summary

The endothelin (ET) and prorenin/renin/prorenin receptor (PRR) systems have opposing physiological effects on collecting duct (CD) salt and water reabsorption. It is unknown if the CD ET and renin/PRR systems interact, hence we examined the effects of deleting CD renin or nephron PRR on CD ET system components. PRR knockout (KO) mice were polyuric and had markedly increased urinary ET-1 and inner medullary CD (IMCD) ET-1 *mRNA*. PRR KO mice had greatly increased IMCD ETA receptor *mRNA* and protein, while ETB *mRNA* and protein were decreased. Water loaded wild-type mice with similar polyuria as PRR KO mice had modestly increased urinary ET-1 excretion and inner medullary ET-1 *mRNA*, while inner medullary ETA and ETB *mRNA* or protein expression were unaffected. In contrast to PRR KO, CD prorenin/renin KO did not alter ET system components. Taken together, these results suggest that the nephron PRR is involved in regulating CD ET system expression, but this effect may be independent of CD-derived renin.

Key words

Endothelin • Prorenin • Collecting duct • Knockout • Receptor

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Introduction

The collecting duct (CD) contains several autocrine and paracrine systems that modulate Na^+ transport. Many of these systems inhibit CD Na^+ and water reabsorption, amongst which the endothelin (ET) system has emerged as being of particular importance. Endothelin-1, which is produced in relatively large amounts by the CD, acts *via* endothelin B (ETB) and possibly endothelin A (ETA) receptors to potently and directly inhibit epithelial Na^+ channel (ENaC) activity and vasopressin-stimulated water permeability (Kohan *et al.* 2011). In contrast, relatively few autocrine and/or paracrine systems are known to stimulate CD Na^+ and water reabsorption (Pearce *et al.* 2015). However, recent studies have implicated the renin/prorenin receptor (PRR) pathway as a potential enhancer of CD Na^+ and water transport (Ramkumar and Kohan 2016). This system involves prorenin, and to a lesser extent renin, binding to the PRR and exerting direct effects independent of angiotensin-II (Ang II); prorenin stimulation of the PRR directly increases ENaC activity and membrane aquaporin-2 expression (Ramkumar and Kohan 2016). Since, within the nephron, prorenin/renin are uniquely synthesized by the CD, and the PRR is expressed in greatest abundance within the CD (Advani *et al.* 2009), the possibility exists that the CD renin/PRR system interacts with the CD ET system; such crosstalk between opposing autocrine/paracrine systems might serve to modulate one another's activity. To date, however, there is no information to our knowledge on cross-regulation between the ET and the renin/PRR systems in the CD. Studies in non-CD cells have demonstrated that ET-1 directly inhibits agonist-stimulated renin synthesis and release (juxtaglomerular apparatus cells) (Kohan *et al.* 2011), however there are no reports in any cell type examining renin/PRR system (independent of Ang II) regulation of the ET system. Consequently, the current study was undertaken to determine if the renin/PRR

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system is capable of modulating the ET system in the CD.

Methods

Animal care

All animal studies were conducted with the approval of the University of Utah Animal Care and Use Committees in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of cell-specific KO mice

Details about generation of nephron-specific PRR KO and CD-specific renin KO mice have been published (Ramkumar *et al.* 2015, Ramkumar *et al.* 2014). All mice were bred on a C57BL/6J background derived from an in-house colony. Wild-type mice were C57BL/6J with no engineered genetic modifications. Nephron-specific PRR KO mice are homozygous for loxP-flanked (floxed) exon 2 of the *ATP6AP2* gene, hemizygous for the Pax8-rtTA transgene (contains the reverse tetracycline transactivator under control of the paired box gene 8 promoter) and hemizygous for the LC-1 transgene (encodes tetracycline-inducible bicistronic Cre recombinase and luciferase) (Ramkumar *et al.* 2015). To induce nephron-wide KO, PRR KO were given 2 mg/ml doxycycline in 2 % sucrose drinking water starting at 1 month of age and continued daily for 12 days, followed by 4 weeks off doxycycline before study. Control mice were the same genotype but did not receive doxycycline. Collecting duct-specific renin KO mice are homozygous for floxed exon 1 of the *Ren1* gene (C57BL/6J mice do not contain the *Ren2* gene) and hemizygous for Cre recombinase under the control of the aquaporin-2 (AQP2) promoter (Ramkumar *et al.* 2014). Controls consisted of mice homozygous for the floxed *Ren1* gene but lacking the AQP2-Cre transgene. Mice from both sexes (1:1) were used for all studies. Genotyping of PRR KO, renin KO and control mice was performed as previously described (Ramkumar *et al.* 2015, Ramkumar *et al.* 2014).

Metabolic cage studies

The PRR KO, renin KO and control mice at 3 months of age were placed in metabolic cages for two consecutive days with free access to a gelled diet with normal sodium (0.3 %) content and drinking water. Water intake and urine volume were recorded on both days; only data from the second day was used since mice typically take a day to acclimate to the cages. Urine osmolality was measured using Osmett II (Precision System, Natick, MA, USA). Urinary ET-1 was determined by enzyme immunoassay (R&D Systems, Minneapolis, MN, USA).

For water loading studies, wild-type mice were placed in metabolic cages for 2 days as described above, fed a normal sodium diet and drinking water, and urine collected on day 2. Mice were removed from metabolic cages and given 3 days rest. Mice were then returned to metabolic cages and started on water loading (normal sodium gelled diet plus free access to drinking water containing 5 % sucrose). Mice were housed in metabolic cages on days 1 and 2, and days 6 and 7 of water loading (mice were not continuously housed in metabolic cages for all 7 days of water loading to avoid undue stress). Water intake, urine volume, urine osmolality and urine ET-1 were determined from days 2 and 7 of water loading (days 1 and 6 not recorded to allow mice a day to acclimate each time they were placed in metabolic cages).

RNA analysis

Messenger RNA levels were determined for ET-1, ETA and ETB from PRR KO and control mouse renal medulla and inner medullary CD (IMCD), renin KO and control mouse inner medulla, and wild-type mice inner medulla (all mice fed a normal sodium diet). In addition, ET-1, ETA and ETB *mRNA* levels in inner medulla of wild-type mice on days 2 and 7 of water loading were determined. RNA was isolated using the PureLink RNA kit (Ambion, Foster City, CA, USA) and reverse transcribed using 0.5 μg of total RNA with oligo(dt) and Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen, Grand Island, NY, USA). The resulting cDNA was assayed for relative expression of ET-1, ETA, ETB and GAPDH *mRNA* using Taqman Gene Expression Assays (ET-1 probe cat # Mm00438656_m1, ETA probe cat # Mm01243722_m1, ETB probe cat # Mm00432989_m1, GAPDH probe cat # Mm03302249_g1, Applied Biosystems, Carlsbad, CA, USA).

Immunofluorescence

Kidneys from control and PRR KO mice were fixed overnight in 10 % formaldehyde, embedded in paraffin and 4 μm sections obtained. Deparaffinized kidney sections were treated with 0.1 % Triton for 10 min for antigen retrieval, blocked with Odyssey blocking

buffer (Licor, Lincoln, NE, USA) for 1 h and incubated with primary antibody against ETA (1:25, Alomone, Jerusalem, Israel) and AQP2 (1:200, Santa Cruz, Dallas, TX, USA) overnight. After 3 consecutive washes of 5 min each with phosphate-buffered saline (PBS), kidney sections were incubated with secondary donkey anti- rabbit Alexa Fluor 488 (1:200) and donkey anti-goat Alexa Fluor 555 (1:400) antibodies for 60 min. After 3 wash-rinse steps of 5 min each with PBS, slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and sealed with a coverslip. Tissue sections were examined and photographed with a Nikon FXA epiflourescence microscope.

Western analysis

Renal inner medullas were dissected and homogenized in ice-cold buffer (50 mM Tris, 50 mM EDTA, 1 % Triton, 1 mM PMSF) and Complete Protease Inhibitors (Roche, St. Louis, MO, USA). Whole cell lysate protein content was determined using the modified Lowry assay and samples were solubilized with Laemmli loading buffer containing 0.5 % lithium dodecyl sulfate. Loading control gels were initially run on 12 % Bis-Tris gels, stained with Coomassie blue and random bands quantified by densitometry to assess equal loading. Equal amounts of protein (10 µg/lane) were run on a denaturing NUPAGE 4-12 % Bis-Tris minigel (Invitrogen) and transferred to a polyvinylidene difluoride plus nylon membrane. Membranes were incubated with antibodies against ETA (1:100, Alomone catalog # AER-001) or ETB (1:200, Alomone catalog # AER-002) or mouse monoclonal β actin (1:1000, Life Technologies, Carlsbad, CA, USA catalog # AM4302). Secondary horseradish peroxidase-conjugated antibodies (goat anti-rabbit for ETA/B catalog # sc-2004 and goat anti-mouse for β actin catalog # sc-2005, Santa Cruz, Dallas, TX, USA) were used at a dilution of 1:2000. Immunoblots were visualized with the Advance ECL system (GE Healthcare, Piscataway, NJ, USA). Densitometry was performed with a Bio-Rad gel documentation system (Hercules, CA, USA).

Inner medullary collecting duct isolation

Mouse IMCD were isolated as previously described (Strait *et al.* 2010). Briefly, mouse renal inner medullas were minced and incubated at 37 °C in 0.1 % collagenase (type I; Worthington, Freehold, NJ, USA) containing 0.01 % DNase (type I) in Hanks' balanced salt solution (HBSS) +15 mM HEPES (pH 7.4). After ~45 min, the digest containing mainly single cells and tubules was filtered through a 74-µm mesh, centrifuged, and suspended in 10 % bovine serum albumin in HBSS, followed by two centrifuge/washes with HBSS. The final pellet containing primarily tubules was suspended in HBSS + HEPES.

Statistics

Comparisons between KO and their relevant control mice, as well as between water loaded and non-water loaded mice, were analyzed by the Student's t-test (unpaired, 1 tail). Data are expressed as mean ± standard error and $p<0.05$ was taken as significant.

Results

PRR KO mouse studies

A hypothesis of this study was that CD PRR modulates the CD ET system. To ideally test this *in vivo*, one would use CD-specific PRR targeted mice; while such mice have been developed, the deficiency of PRR during embryogenesis is cause for concern since subtle or gross developmental abnormalities can occur (Song *et al.* 2013). To avoid these developmental issues, mice were utilized with doxycycline-inducible PRR KO, thereby permitting gene targeting during adulthood (doxycycline started at 1 month of age in the current study). These mice have whole nephron gene targeting (Pax8 promoter drives Cre recombinase in the proximal tubule through the IMCD), hence they have PRR deleted throughout the entire nephron (no inducible CD-specific PRR targeting mice exist).

PRR KO mice have been reported to have, under physiological conditions, major alterations in water, and modest changes in sodium, metabolism (Ramkumar *et al.* 2015, Ramkumar *et al.* 2016). To confirm this, PRR KO and control mice were studied in metabolic cages; PRR KO mice had higher water intake, higher urine volume (3-4 fold), and reduced urine osmolality compared to controls (Figs 1A1-1A3) under baseline conditions (normal sodium intake and free access to drinking water). Under these same conditions, PRR KO mice had marked elevated urinary ET-1 excretion (~10-fold vs. controls) (Fig. 2A1) and inner medullary ET-1 *mRNA* levels (Fig. 2A2); the inner medulla was the focus of analysis since it contains predominantly CDs. PRR KO mice had a pronounced increase in inner medullary ETA *mRNA* (~10-fold vs. controls) and reduced ETB *mRNA* (0.6-fold) compared to controls (Fig. 2A2). Similar to the *mRNA*

levels, PRR KO mice had greatly increased inner medullary ETA protein (~20-fold vs. controls) and decreased inner medullary ETB protein compared to controls (Figs 3A1 and 3A2). To help support the notion that PRR KO modulated CD ET receptors, kidneys from PRR KO and control mice were immunostained. We were unable to obtain reliable ETB immunostaining with a variety of ETB antibodies, however ETA immunostaining was detected: there was a marked increase in CD ETA immunostaining in PRR KO mice compared to controls (sections co-labeled for aquaporin-2 to identify CDs) (Fig. 4). Note that these images show outer medulla since we were unable, for unclear reasons, to get consistent staining of inner medulla. To further confirm that IMCD was involved, IMCD were acutely isolated and ET system *mRNA* determined (protein levels too low to use Western analysis). As shown in Figure 4, IMCD from PRR KO mice had an identical *mRNA* pattern as seen in inner medulla – increased ET-1 and ETA *mRNA*, and reduced ETB *mRNA*, as compared to controls.

Renin KO mouse studies

Collecting duct-specific renin KO mice were examined in order to determine whether CD-derived renin modulates the collecting duct ET system. We have previously reported on these mice (Ramkumar *et al.* 2014), however to reassess urine water excretion, the major factor altered in PRR KO mice, renin KO mice were subjected to metabolic cage studies. No differences in water intake, urine volume or urine osmolality were observed between renin KO and control mice (Figs 1B1-1B3); although not reported herein, renin KO mice also do not have altered urine Na excretion compared to controls under physiological conditions (Ramkumar *et al.* 2014). Renin KO mice had no difference in urinary ET-1 excretion (Fig. 2B1) or inner medullary ET-1 *mRNA* (Fig. 2B2) compared to controls on a normal sodium diet with free access to drinking water. Renin KO mice also had no alteration in ETA or ETB *mRNA* (Fig. 2B2) or protein (Figs 3B1 and 3B2) compared to controls.

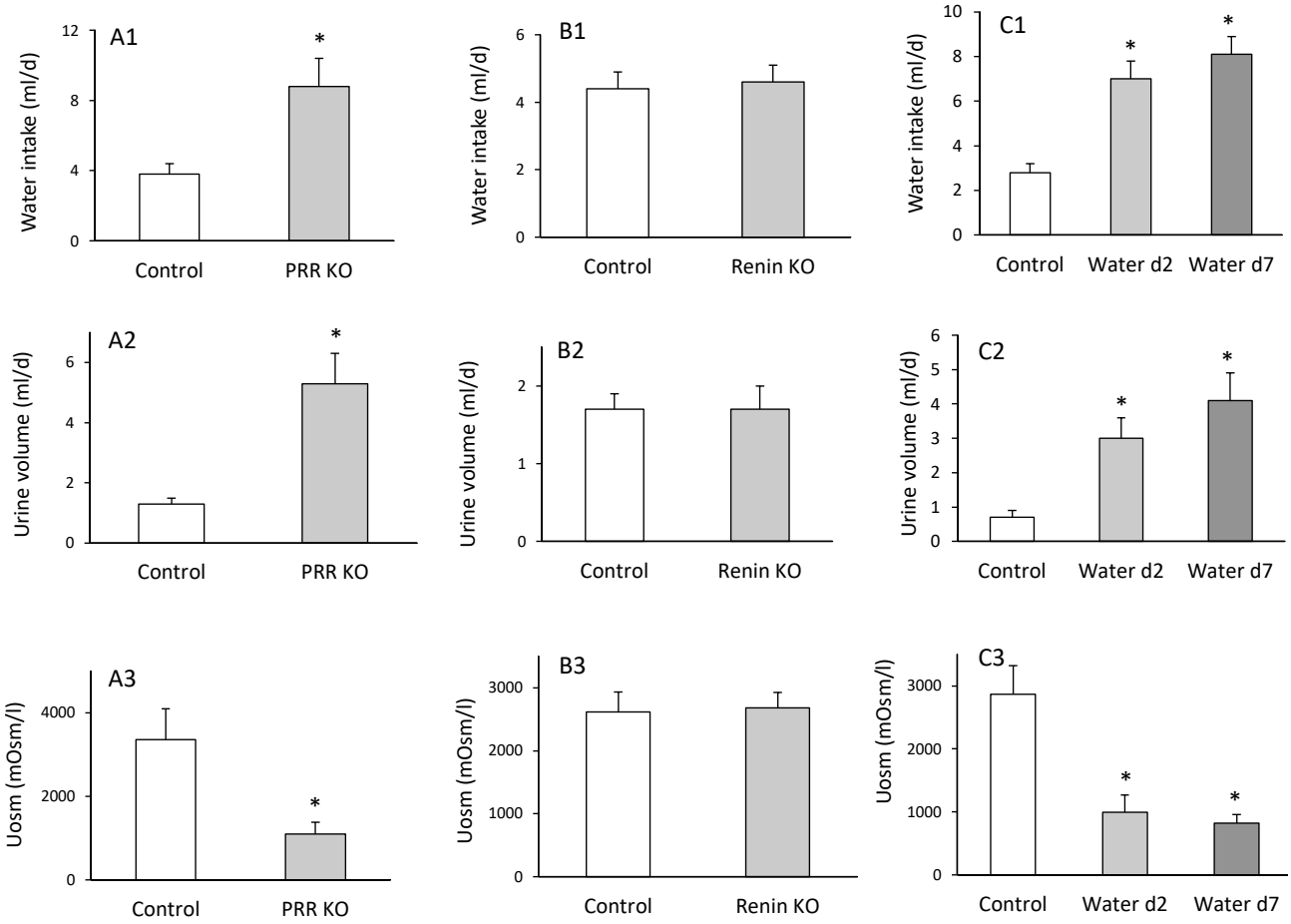

Fig. 1. Effect of nephron-specific PRR KO, collecting duct-specific renin KO and water loading in WT mice on water intake (**A1-C1**), urine volume (**A2-C2**) and urine osmolality (**A3-C3**). Control and PRR/renin KO mice were fed a normal sodium (0.3 %) diet and given free access to drinking water. WT mice were fed a normal sodium (0.3 %) diet and given free access to drinking water containing 5 % sucrose for 2 or 7 days; controls were given drinking water without sucrose. N=6 each data point. *p<0.05 vs. control.

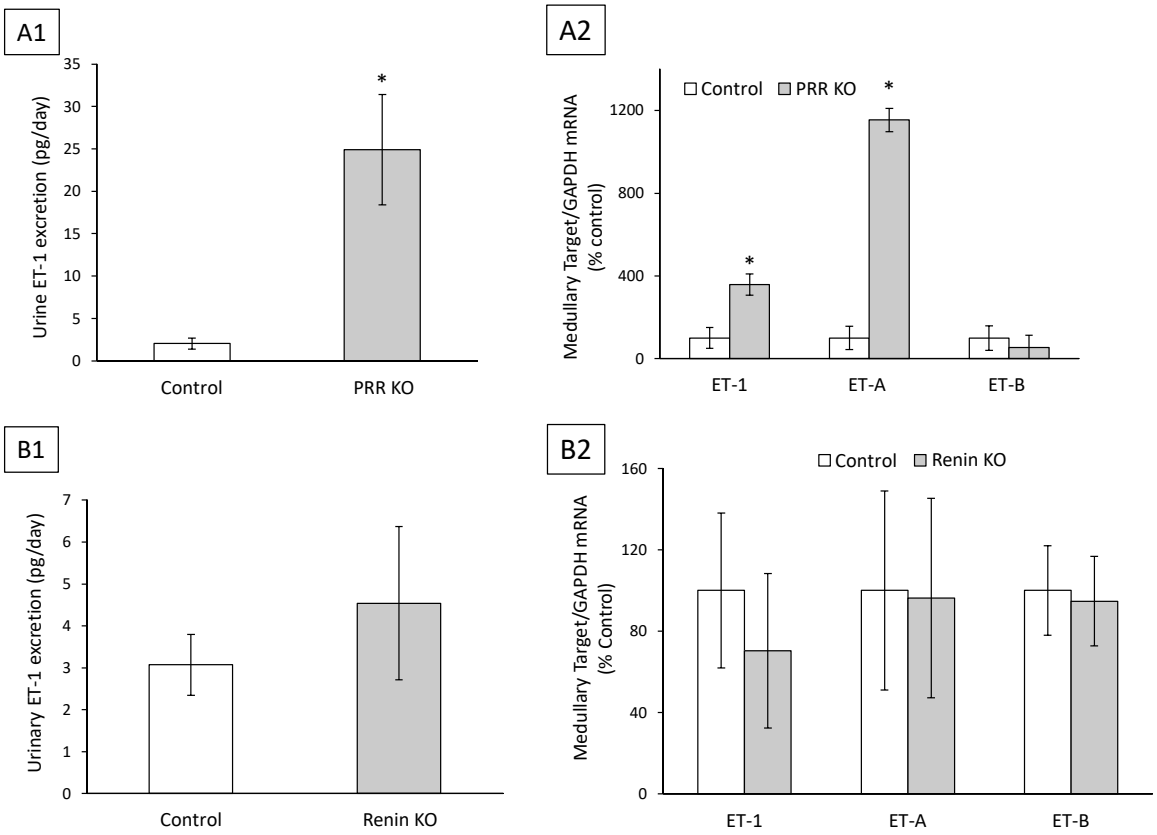

Fig. 2. Effect of nephron-specific PRR KO or collecting duct-specific renin KO on urinary ET-1 excretion (**A1-B1**) and inner medullary ET-1, ETA and ETB receptor *mRNA* content (**A2-B2**). Control and PRR/renin KO mice were fed a normal sodium (0.3 %) diet and given free access to drinking water. N=6 each data point. *p<0.05 vs. control.

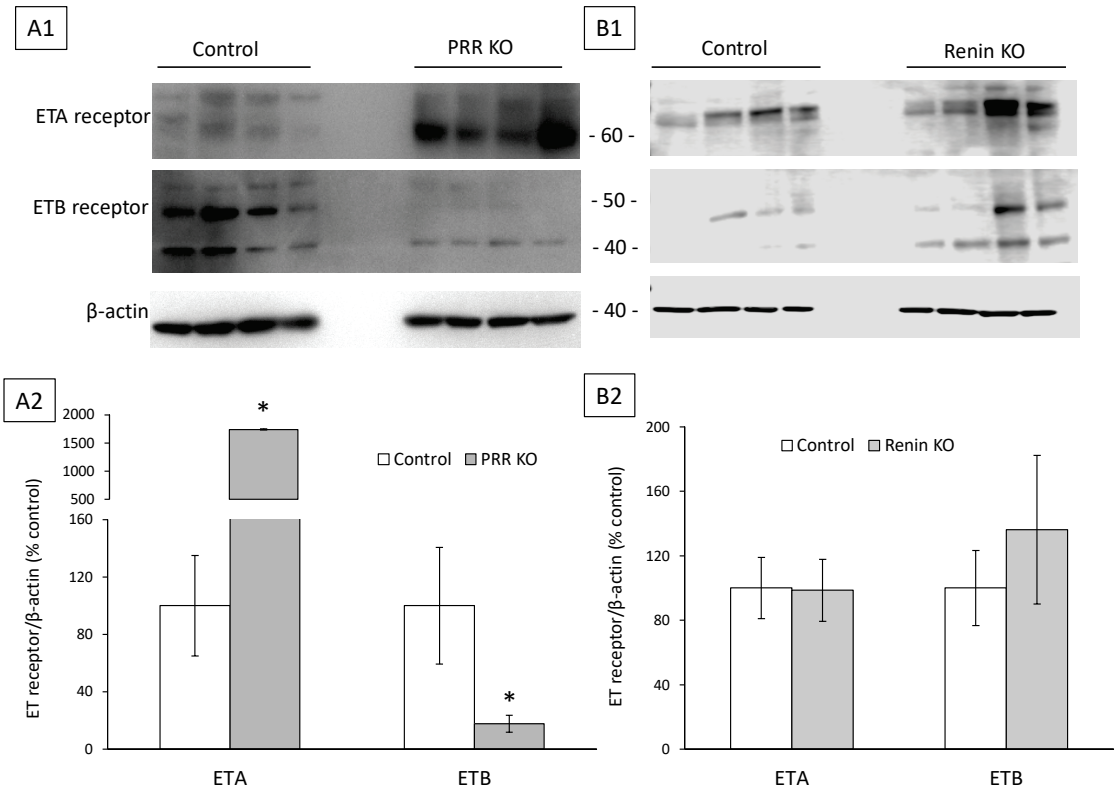

Fig. 3. Effect of nephron-specific PRR KO or collecting duct-specific renin KO on inner medullary ETA and ETB receptor protein content. The Western blot is shown in Panel **A1** and **B1**, and densitometry data in Panel **A2** and **B2**. Control and PRR/renin KO mice were fed a normal sodium (0.3 %) diet and given free access to drinking water. N=4 each data point. *p<0.05 vs. control.

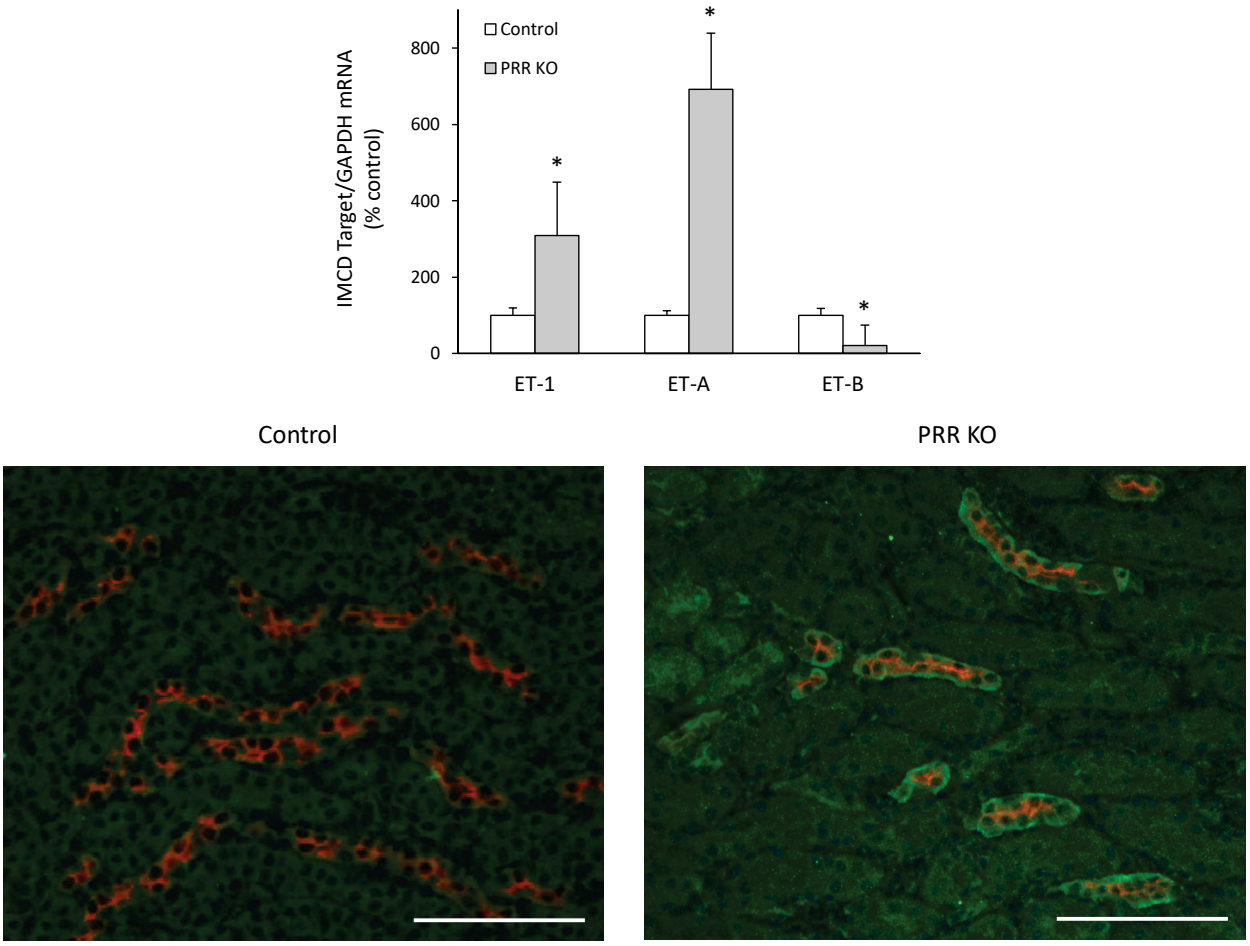

Fig. 4. Effect of nephron-specific PRR KO on acutely isolated mouse inner medullary collecting duct (IMCD) ET-1, ETA and ETB receptor *mRNA* content (**upper panel**) and ETA receptor immunostaining. IMCD were obtained from control and PRR KO mice fed a normal sodium (0.3 %) diet and given free access to drinking water. N=6 each data point. *p<0.05 vs. control. Renal medullary sections were immunostained for aquaporin-2 (red) and ETA receptor (green). Representative figures are shown from 6 separate mice with multiple sections stained in each mouse. Scale bar = 100 μm.

Water loading studies

The effect of water loading on renal ET system components was examined since PRR KO mice are polyuric, and diuresis has been associated with increased urinary ET-1 excretion (Lyon-Roberts *et al.* 2011). Administration of 5 % sucrose in drinking water *ad libitum* resulted in water intake, urine volume and urine osmolality similar to that seen with PRR KO mice (Fig. 1C1-1C3). ET system responses were assessed on days 2 and 7 of water loading to help evaluate acute vs. chronic responses (note that it is not possible to get reliable data from day 1 of water loading since the mice need a day to adjust to the sucrose drinking water). As expected, urinary ET-1 excretion increased on both days of water loading (Fig. 5A), although to a much smaller degree than that seen with PRR KO mice (Fig. 2A1). Similarly, both days of water loading increased inner medullary ET-1 *mRNA* (Figs 5B and 5C), although again less than that observed in PRR KO mice (Fig. 2A2). Water loading (days 2 and 7) did not alter inner medullary ETA or ETB *mRNA* (Figs 5B and 5C) or protein (Figs 6A-6D).

Discussion

The current study demonstrates, for the first time, an interaction between the PRR and the CD ET systems. Mice with deletion of the nephron PRR had increased IMCD ET-1 and ETA, and decreased renal medullary ETB, expression. In contrast, CD renin KO mice had no alterations in expression of CD ET system components. Urinary ET-1 excretion was markedly enhanced in nephron PRR KO mice while it was unchanged in CD renin KO mice. Collectively, these findings suggest that the nephron PRR, but not CD-derived renin, regulates the CD ET system.

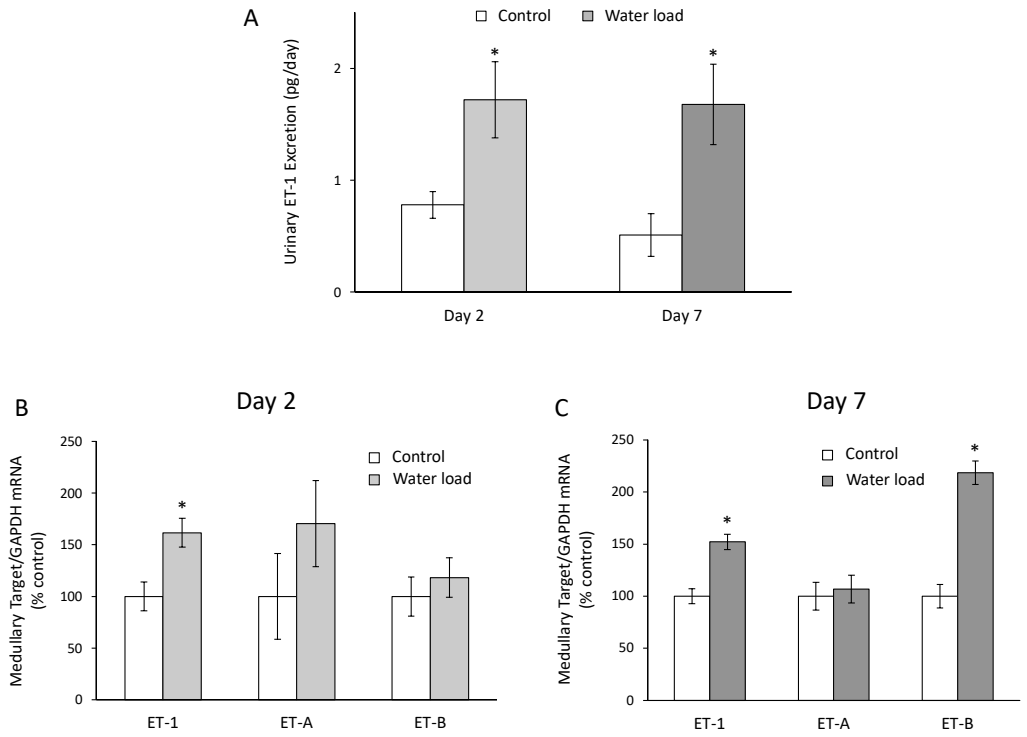

Fig. 5. Effect of water loading on urinary ET-1 excretion (**A**) and inner medullary ET-1, ETA and ETB receptor *mRNA* content in wild-type mice. Mice were fed a normal sodium (0.3 %) diet and given free access to drinking water containing 5 % sucrose for 2 days **(B)** or 7 days **(C)**; controls were given drinking water without sucrose. N=6 each data point. *p<0.05 vs. control.

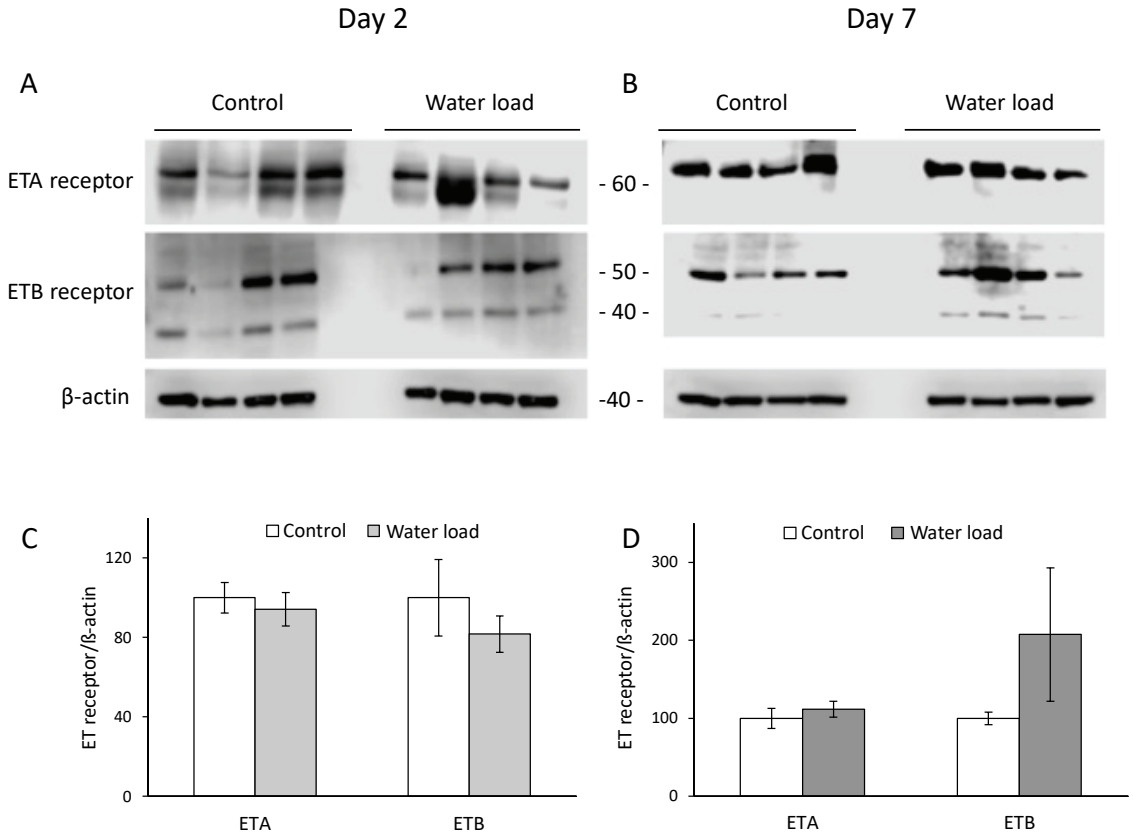

Fig. 6. Effect of water loading on inner medullary ETA and ETB receptor protein content in wild-type mice. Mice were fed a normal sodium (0.3 %) diet and given free access to drinking water containing 5 % sucrose for 2 (**A** and **C**) or 7 (**B** and **D**) days; controls were given drinking water without sucrose. Western blots are shown in **A** and **B** and densitometry data for these blots shown in **C** and **D**, respectively. N=4 each data point.

One potential explanation for ET system changes in nephron PRR KO mice relates to increased tubule and urinary flow. Previous studies have shown increased urinary ET-1 excretion following salt or water loading (Lyon-Roberts *et al.* 2011). This was confirmed in the present study; however the magnitude of the elevated CD and urinary ET-1 levels following water loading in wild-type mice was much less than that observed in nephron PRR KO mice. Moreover, medullary ETA and ETB expression were unaffected by water loading in wild-type mice, but were markedly altered in PRR KO mice (notably, the effect of water loading on renal medullary ET receptor expression to our knowledge has not been previously reported). Hence, it is unlikely that the diuresis in nephron PRR KO mice is directly responsible for the alterations in the CD ET system.

Another possibility is that PRR-induced signaling modulates the CD ET system. One way this could occur is through activation of PRR by renin or prorenin. We found no evidence that CD-derived prorenin or renin regulated the CD ET system; however, since filtered renin can reach the urine (Roksnoer *et al.* 2016) and urinary renin is still present (albeit reduced) in CD renin KO mice (Ramkumar *et al.* 2014), the possibility cannot be excluded that renin/prorenin binding to PRR is involved in PRR regulation of the CD ET system. Most simply, this could involve PRR activation of prorenin to promote formation of Ang II. However, although Ang II increases ET-1 production by several cell types, it was shown to either not change, or in combination with high salt intake to decrease, inner medullary ET-1 content (Sasser *et al.* 2002). Interestingly, Ang II increases urinary ET-1 excretion (Sasser *et al.* 2002). Further, Ang II decreased ETA and ETB expression in isolated IMCD (Wong and Tsui 2001). PRR signaling independent of Ang II could also potentially be involved in modulating the ET system. Prorenin activation of PRR on CD cells can induce prostaglandin E_2, protein kinase A, nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), serum and glucocorticoid-regulated kinase 1 (SGK1), the Wnt/β-catenin pathway, cyclooxygenase-2 and extracellular signal-regulated kinase 1/2 (ERK1/2) (Advani *et al.* 2009, Gonzalez *et al.* 2012, Lu *et al.* 2016a, Lu *et al.* 2016b, Wang *et al.* 2016). Amongst the above, CD ET-1 can be stimulated by NOX4 (Wheatley and Kohan 2017) and SGK1 (Gumz *et al.* 2003); however this would not explain why PRR KO increases CD ET-1. In contrast to ET-1, beyond Ang II regulation discussed above, little is known about factors controlling ET receptor expression in the CD. One possibility is that PRR deficiency in the nephron PRR KO mice reduces autophagosomal function (Trepiccione *et al.* 2016) leading to increased accumulation of at least ETA (would not explain the decrease in ETB). However, ETA *mRNA* is increased in nephron PRR KO mice, so at least a transcriptional and/or translational effect is involved.

The physiological significance of PRR differential regulation of CD ETA and ETB is unknown. As discussed earlier, one might speculate that the PRR and ET systems interact to regulate renal salt and/or water excretion. In general, under physiological conditions, the natriuretic and diuretic effects of ET-1 on the CD are thought to be primarily due to ETB receptor activation, albeit ETA receptors have also been implicated (reviewed in Kohan *et al.* 2011, Lynch *et al.* 2013, Stuart *et al.* 2013); it is unclear what the net effect of PRR would be on ET system activation with respect to salt and water excretion. Further, it is unclear if the effects of PRR KO on ET receptors are separately mediated or if primary modulation of one receptor might induce compensatory changes in the other (e.g. PRR signaling may directly affect ET-1 and ETA with secondary compensatory changes in ETB). Another possibility is that PRR regulates the CD ET system for purposes independent of renal salt and/or water excretion. Both PRR (Li *et al.* 2017) and ET-1 (*via* ETA) (Culshaw *et al.* 2015) have been shown to promote renal pro-fibrotic pathways, while renal ETB may exert anti-fibrotic effects (Forbes *et al.* 2001). Hence, it may be that PRR activation that favors fibrosis may lead to downregulation of CD ET-1/ETA and upregulation of CD ETB as compensatory antifibrotic responses. Clearly, further investigation of these possibilities is needed, albeit beyond the scope of the current study.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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