Testosterone increases urinary calcium excretion and inhibits expression of renal calcium transport proteins

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Although gender differences in the renal handling of calcium have been reported, the overall contribution of androgens to these differences remains uncertain. We determined here whether testosterone affects active renal calcium reabsorption by regulating calcium transport proteins. Male mice had higher urinary calcium excretion than female mice and their renal calcium transporters were expressed at a lower level. We also found that orchidectomized mice excreted less calcium in their urine than sham-operated control mice and that the hypocalciuria was normalized after testosterone replacement. Androgen deficiency increased the abundance of the renal mRNA and protein of both the luminal transient receptor potential vanilloid-subtype 5 (TRPV5) and intracellular calbindin-D_{28K} transporters, which in turn were suppressed by testosterone treatment. There were no significant differences in serum estrogen, parathyroid hormone, or 1,25-dihydroxyvitamin D3 levels between control and orchidectomized mice with or without testosterone. Moreover, incubation of primary rabbit connecting tubule and cortical collecting duct cells with a nonaromatizable androgen, dihydrotestosterone, reduced transcellular calcium transport. Thus, our study shows that gender differences in renal calcium handling are, in part, mediated by the inhibitory actions of androgens on TRPV5-mediated active renal calcium transport.

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Several studies have reported sex differences in the urinary Ca^{2+} excretion, showing a greater urinary Ca^{2+} loss in male mice than in female mice.[1,2] In addition, estrogens have been shown to increase the renal reabsorption of Ca^{2+}, which is in good agreement with the observed gender differences.[3] Presently, it remains unclear whether androgens have an opposing role to estrogens in modulating renal Ca^{2+} reabsorption. The androgen receptor (AR) is expressed in renal epithelial cells,[4] and a growing body of evidence points to sex differences in various functional characteristics of mammalian kidneys; for example, a higher glomerular filtration rate in the male rat kidney (possibly due to higher renal plasma flow and lower vascular resistance) (reviewed in[5]). However, the role of androgens in regulating renal Ca^{2+} handling remains poorly characterized.

In the kidney, Ca^{2+} reenters the blood by passive paracellular as well as active transcellular reabsorption. The active Ca^{2+} reabsorptive component is restricted to the distal convoluted tubules and the connecting tubules (CNTs).[6–9] Here, Ca^{2+} enters the epithelial cell through the Ca^{2+}-selective ion channel transient receptor potential vanilloid-subtype 5 (TRPV5). Subsequently, Ca^{2+} is bound to calbindin-D_{28K} that transports Ca^{2+} from the apical to the basolateral side where the Na^+/Ca^{2+}-exchanger (NCX1) and the plasma membrane ATPase (PMCA1b) extrude Ca^{2+} into the peritubular lumen.[6]

Active renal Ca^{2+} reabsorption is critical in determining the final urinary Ca^{2+} excretion, and has been shown to be regulated by calciotropic hormones, including parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).[6,10,11] Estrogen has also been shown to affect active renal Ca^{2+} transport, although sex hormones are usually not considered as calciotropic factors.[3,6,12]

This study aims to determine whether androgens contribute to the gender differences in renal Ca^{2+} handling by regulating the expression of Ca^{2+} transport proteins TRPV5, calbindin-D_{28K}, NCX1, and PMCA1b. Because changes in systemic androgen concentrations also affect bone mineralization through a long-term process,[13] we evaluated the

short-term effects of androgen deficiency in orchidectomized (ORX) mice and of testosterone resupplementation (ORX + T), on the expression of the renal Ca^{2+} transporters. In addition, to exclude possible effects of androgens on bone turnover, we applied nonaromatizable dihydrotestosterone (DHT) to an isolated cell system of primary renal CNT/cortical collecting duct (CCD) cells.

RESULTS

Gender differences in urinary Ca^{2+} excretion

To investigate whether sex differences could be noted in renal and intestinal Ca^{2+} handling, we determined 24 h urinary Ca^{2+} excretion and intestinal absorption in age-matched male and female mice. Body weight (26.7 ± 0.8 g in male vs. 25.4 ± 1.8 g in female) and diuresis (1.3 ± 0.5 ml/24 h in male vs. 1.1 ± 0.3 ml/24 h in female) was not significantly different between sexes. Male mice showed a significant higher 24 h urinary Ca^{2+} excretion (4.1 ± 0.3 vs. 3.3 ± 0.2 µmol per day) and Ca^{2+}/creatinine (Cr) ratio (0.23 ± 0.03 vs. 0.18 ± 0.02), in comparison to females (Figure 1a). Male and female mice consumed similar amounts of food (3.30 ± 0.02 vs. 3.20 ± 0.03 g, respectively) and hence ingested comparable amounts of Ca^{2+} (33.0 ± 0.2 vs. 32.4 ± 0.3 mg, respectively). In addition, intestinal radioactive Ca^{2+} tracer uptakes were performed in male and female mice. Intestinal Ca^{2+} absorption was determined by an *in vivo* absorption assay, measuring serum levels of radioactive $^{45}Ca^{2+}$ at several time

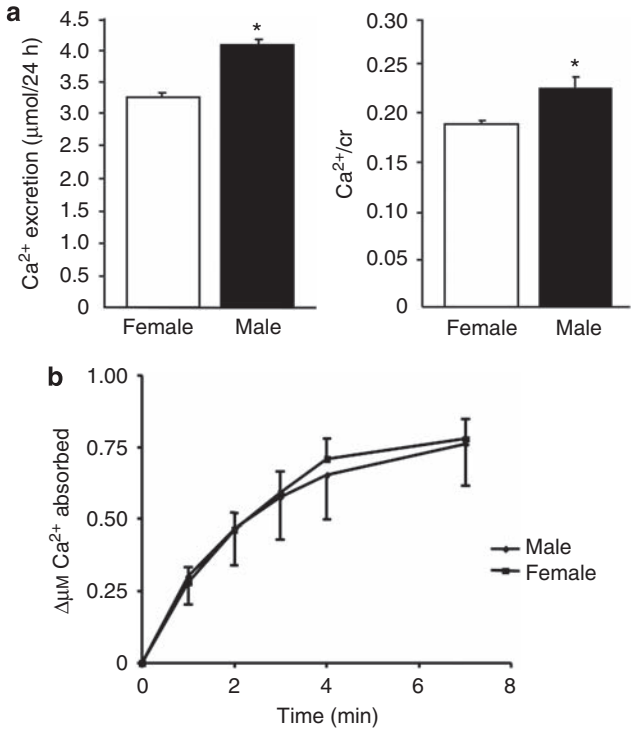

Figure 1 | Mouse sex differences in urinary Ca^{2+} excretion. (**a**) 24 h urine Ca^{2+} excretion and Ca^{2+}/Cr ratio were determined in both female and male mice. Data are presented as means ± s.e.m. Cr, creatinine. *$P < 0.05$ male vs. female mice. $n = 8$ samples per group. (**b**) Intestinal $^{45}Ca^{2+}$ absorption into serum of male (♦) and female (■) mice after oral gavage. $n = 5$ animals per group.

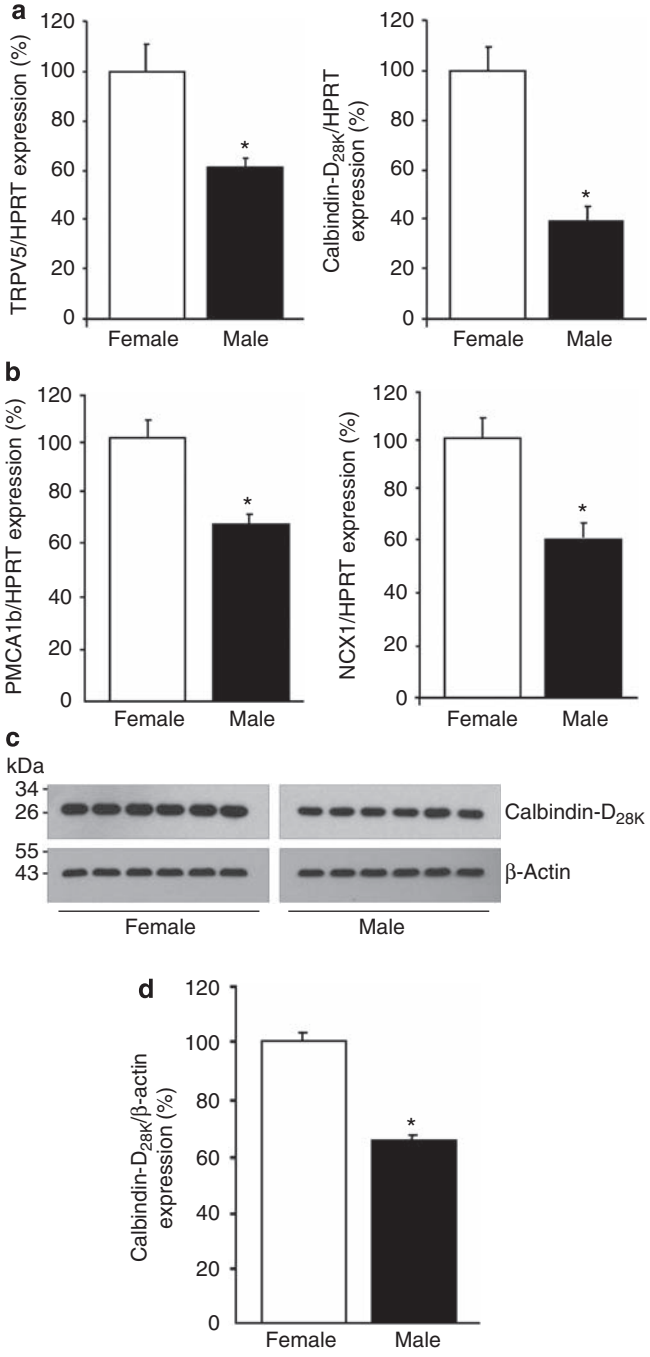

Figure 2 | Mouse sex differences in the expression of renal Ca^{2+} transporters. (**a**) Renal mRNA expression of transient receptor potential vanilloid-subtype 5 (TRPV5) and calbindin-D_{28K} were determined by real-time quantitative RT-PCR analysis, expressed as the ratio of hypoxanthine-guanine phosphoribosyl transferase (HPRT) and depicted as percentage of female mice. (**b**) Similarly, mRNA expression of plasma membrane ATPase (PMCA1b) and Na^+/Ca^{2+}-exchanger (NCX1) were determined in kidney RNA isolates from male and female mice. mRNA expression was corrected for endogenous HPRT. (**c**) Immunoblots of protein samples (10 µg each) from homogenates of kidney tissues were labeled with antibodies against calbindin-D_{28K} or β-actin. (**d**) Expression of calbindin-D_{28K} protein was quantified by computer-assisted densitometry analysis and presented as the ratio to β-actin expression levels, in relative percentages compared with female mice. Data are presented as means ± s.e.m. *$P < 0.05$ male vs. female mice. $n = 6$ samples per group.

points after oral gavage. The intestinal absorption of Ca^{2+} was similar in both males and females (Figure 1b).

Sex differences in renal expression of Ca^{2+} transporters

The increased urinary Ca^{2+} excretion and Ca^{2+}/Cr ratio in male mice was paralleled by a significant decline in the renal mRNA expression of TRPV5, calbindin-D_{28K}, NCX1, and PCMA1b (Figure 2a, b). For calbindin-D_{28K} abundance, this was confirmed by immunoblotting (Figure 2c). Densitometrical analysis of the immunoblots showed significantly less calbindin-D_{28K} protein expression in male mice than in female mice (Figure 2d). Similarly, computerized analysis of immunohistochemical images revealed a significant decrease in TRPV5 and calbindin-D_{28K} abundance in male mice as compared to female mice (Figure 3).

Localization of the AR in mouse kidney

Binding of the steroid hormone to the AR may regulate the expression of renal Ca^{2+} transporters. To investigate whether the AR is localized in TRPV5-expressing cells, we performed immunohistochemical labeling of TRPV5 and the AR using mouse kidney sections. As depicted in Figure 4, TRPV5 and the AR are coexpressed in distal convoluted tubule/CNT cells.

Effects of ORX and testosterone treatment on serum and urine parameters

To specifically address the effects of androgens on renal Ca^{2+} handling, we measured urinary Ca^{2+} excretion in sham-operated, ORX mice, and in ORX + T. Importantly, the body weight of mice between sham-operated, ORX, and ORX + T groups was not different (27.1 ± 0.7, 26.1 ± 0.9, and 26.9 ± 0.9 g, respectively). ORX significantly decreased urinary excretion of Ca^{2+} (4.4 ± 0.3 (sham-operated) vs. 2.3 ± 0.2 (ORX) µmol per day) and the Ca^{2+}/Cr ratio (0.28 ± 0.05 (sham-operated) vs. 0.16 ± 0.03 (ORX)) (Figure 5). A change in renal transport was apparent, as the fractional Ca^{2+} excretion was significantly reduced (0.94 ± 0.12 (sham-operated) vs. 0.49 ± 0.04 (ORX)%). Testosterone supplementation of ORX mice restored renal Ca^{2+} excretion (4.3 ± 0.3 µmol per day) and the Ca^{2+}/Cr ratio (0.25 ± 0.03) to values comparable with the sham-operated mice. Table 1 summarizes the effects of ORX and testosterone replacement therapy on systemic Ca^{2+} handling, calciotropic hormones, and sex hormones. Serum testosterone levels were effectively reduced in untreated ORX mice, whereas supplementation with Sustanon 250 resulted in significantly higher serum testosterone levels. Importantly, serum PTH, $1,25(OH)_2D_3$, and estrogen levels were not significantly different in ORX mice as compared to sham-operated and ORX + T mice.

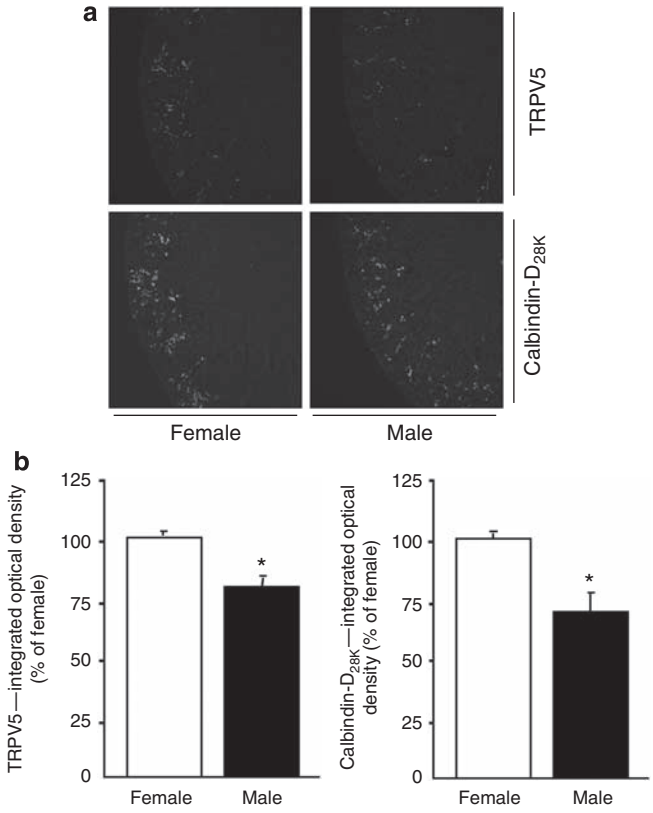

Figure 3 | Gender differences in protein expression of Ca^{2+} transporters in mouse kidney. (**a**) Representative images of immunohistochemical staining of transient receptor potential vanilloid-subtype 5 (TRPV5) and calbindin-D_{28K} in male and female kidney cortex. (**b**) Semiquantification of renal TRPV5 and calbindin-D_{28K} protein abundance was performed by computerized analysis of immunohistochemical images. Data were calculated as integrated optical density (arbitrary units) and depicted as percentage of female mice. Data are presented as means ± s.e.m. *$P < 0.05$ male vs. female mice. $n = 8$ samples per group.

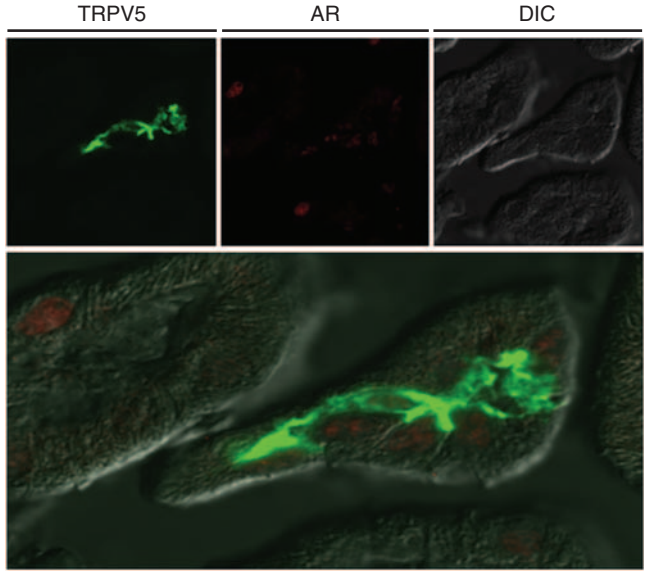

Figure 4 | Characterization of androgen receptor (AR) localization in kidney. Confocal laser microscopy of double-labeled mouse kidney sections using guinea pig anti-transient receptor potential vanilloid-subtype 5 (TRPV5) (TRPV5, upper left panel) and rabbit anti-AR antibodies (AR, upper middle panel). Differential interference contrast (DIC, upper right panel) and overlay (lower panel) are also presented.

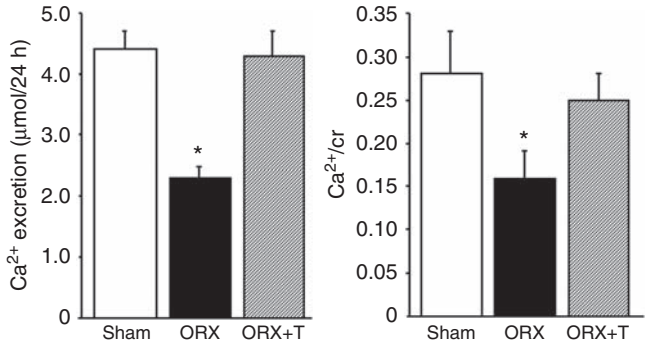

Figure 5 | Differences in urinary Ca^{2+} excretion of sham-operated, orchidectomized (ORX) and of testosterone resupplementation (ORX + T) mice. 24 h urine Ca^{2+} excretion and Ca^{2+}/Cr ratio were determined in sham-operated (Sham), ORX, and ORX + T mice. Data are presented as means ± s.e.m. Cr, creatinine. *$P < 0.05$ male vs. female mice. $n = 8$ per group.

Table 1 | Effect of testosterone on serum Ca^{2+} and calciotropic hormones

	Sham	ORX	ORX+T
Ca^{2+} (mmol/l)	2.8 ± 0.1	2.6 ± 0.1	2.5 ± 0.1
Testosterone (ng/dl)	499 ± 129	59 ± 11[a]	1005 ± 291[a,b]
Estrogen (pg/ml)	63 ± 16	36 ± 12	50 ± 10
PTH (pg/ml)	23.9 ± 6.5	20.7 ± 5.5	25.4 ± 7.4
$1,25(OH)_2D_3$ (pmol/ml)	156 ± 19	130 ± 11	143 ± 17

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone.
Serum concentrations of Ca^{2+} and calciotropic hormones in sham-operated mice and ORX mice with or without testosterone replacement (Sustanon 250, 250 mg/kg per week subcutaneously, 2 weeks).
[a]$P < 0.05$ vs. sham-operated mice. n=8 samples per parameter.
[b]$P < 0.05$ vs. ORX mice. n=8 samples per parameter.

Effects of ORX and testosterone treatment on the expression of renal Ca^{2+} transporters

To address the molecular mechanism responsible for the effect of testosterone on renal Ca^{2+} handling, we examined the expression of TRPV5, calbindin-D_{28K}, PMCA1b, and NCX1 using real-time quantitative reverse transcriptase (RT) PCR, immunoblotting, and immunohistochemistry. ORX mice showed a 3.2-fold increase in TRPV5 and a 2.0-fold increase in calbindin-D_{28K} mRNA expression as compared to sham-operated mice (Figure 6a). Conversely, administration of testosterone to ORX mice (ORX + T) resulted in a significant decrease of TRPV5 and calbindin-D_{28K} mRNA expression (Figure 6a, dashed bars). Similarly, NCX1 was increased in the ORX group, whereas both PMCA1b and NCX1 were decreased in the ORX + T group (Figure 6b). As determined by semiquantitative immunoblotting, protein abundance of calbindin-D_{28K} was increased in ORX mice compared to sham-operated mice (Figure 6c, d). In accordance, calbindin-D_{28K} protein abundance in ORX + T mice was comparable to the sham-operated controls (Figure 6c, d). In line with the above, semiquantification of protein expression, the immunohistochemical labeling experiments revealed a significant upregulation of TRPV5 (2.7-fold) and

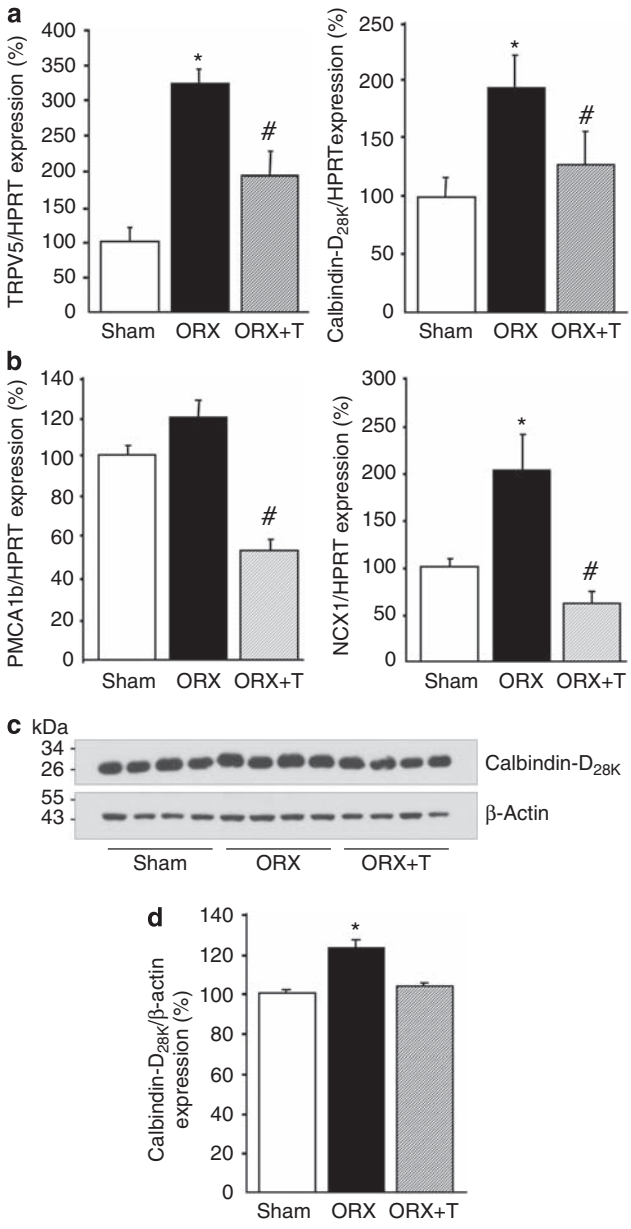

Figure 6 | Effects of ORX and testosterone treatment on renal mRNA expression of Ca^{2+} transporters. (**a**) Renal mRNA expression of transient receptor potential vanilloid-subtype 5 (TRPV5) and calbindin-D_{28K} in sham-operated, ORX, and ORX + T mice were analyzed by quantitative real-time RT-PCR analysis. (**b**) Expression of plasma membrane ATPase (PMCA1b) and Na^+/Ca^{2+}-exchanger (NCX1) in the kidney of sham-operated, ORX, and ORX + T mice. In all cases expression was normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT) and depicted as percentage of sham-operated mice. (**c**) Immunoblots of protein samples (10 μg each) from homogenates of kidney tissue of sham-operated, ORX, and ORX + T mice were labeled with antibodies against calbindin-D_{28K} or β-actin. (**d**) Expression of calbindin-D_{28K} protein was quantified by computer-assisted densitometry analysis and presented as the ratio to β-actin expression levels, in relative percentages compared with sham-operated mice. Data are presented as means ± s.e.m. Sham, sham-operated mice; ORX, orchidectomized mice; ORX + T, orchidectomized mice treated with Sustanon 250 subcutaneously (250 mg/kg per week) for 2 weeks. *$P < 0.05$ vs. sham-operated mice. $P < 0.05$ vs. ORX mice. $n = 8$ samples per group.

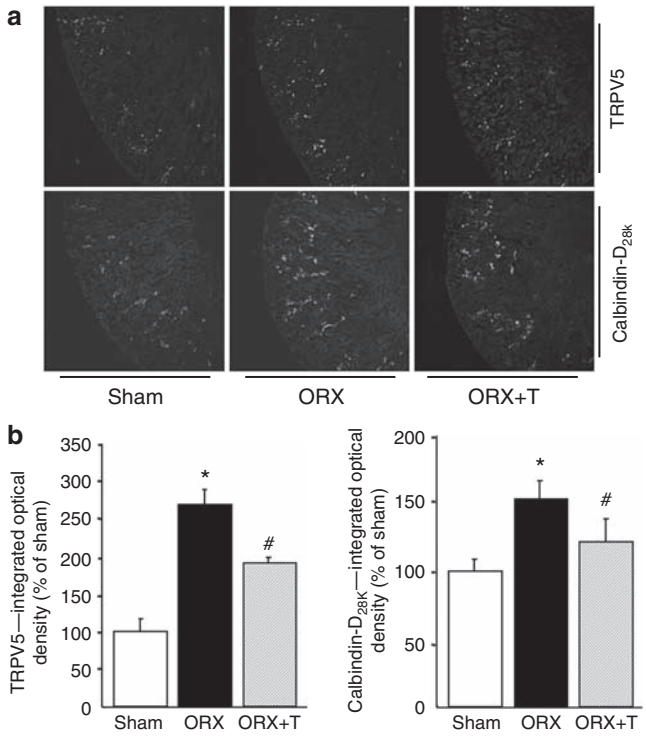

Figure 7 | Immunohistochemical staining of renal Ca^{2+} transporters in sham-operated, ORX, and ORX + T mice. (**a**) Representative images of immunohistochemical staining of transient receptor potential vanilloid-subtype 5 (TRPV5) and calbindin-D_{28K} in kidney cortex of sham-operated, ORX, and ORX + T mice. (**b**) Semiquantification of renal TRPV5 and calbindin-D_{28K} protein abundance was performed by computerized analysis of immunohistochemical images. Data were calculated as integrated optical density (arbitrary units), depicted as percentage of sham-operated mice, and presented as means ± s.e.m. Sham, sham-operated mice; ORX, orchidectomized mice, ORX + T, orchidectomized mice treated with Sustanon 250 subcutaneously (250 mg/kg per week) for 2 weeks. $^{*}P < 0.05$ vs. sham-operated mice. $^{\#}P < 0.05$ vs. ORX mice. $n = 8$ samples per group.

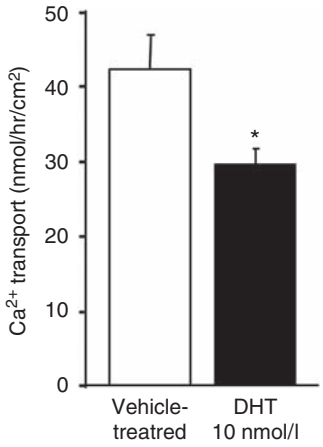

Figure 8 | Effect of dihydrotestosterone (DHT) on net apical to basolateral Ca^{2+} transport in primary cultures of rabbit connecting tubule (CNT)/cortical collecting duct (CCD) cells. Transepithelial Ca^{2+} transport across confluent monolayers was measured in the absence or presence of 10 nmol/l DHT. At the end of the 90 min incubation period, apical medium was collected to determine the amount of Ca^{2+} transport across the monolayer. Transepithelial Ca^{2+} transport is expressed nmol/h/cm^2. Data from three independent experiments ($n = 21$ per condition) were combined. $^{*}P < 0.001$.

calbindin-D_{28K} (1.5-fold) signal in ORX mice when compared to sham-operated mice (Figure 7). Furthermore, treatment of ORX mice with testosterone led to a significant decline in TRPV5 and calbindin-D_{28K} signals (Figure 7).

Effect of DHT on transcellular Ca^{2+} transport in rabbit kidney CNT and CCD primary cell cultures

The effect of androgen on renal Ca^{2+} handling in the ORX mice may be facilitated by the possible interference of other organs (e.g., bone). Therefore, the effect of androgen on TRPV5-mediated Ca^{2+} transport was studied in an isolated renal cell system. Primary cultures of rabbit CNT/CCD cells were grown to confluence on permeable supports. The cells were treated with DHT or vehicle, and the rate of transepithelial Ca^{2+} transport was determined. Application of 10 nmol/l DHT to the polarized confluent cell monolayers for 24 h significantly inhibited the net apical-to-basolateral transport of Ca^{2+} ($P < 0.02$) (Figure 8).

DISCUSSION

This study is to our knowledge the first to delineate the effect of androgens on renal handling of Ca^{2+} and TRPV5-mediated active Ca^{2+} transport. We find that testosterone contributes significantly to the sex differences observed in renal Ca^{2+} handling. This conclusion is based on the following observations: First, male mice have a greater urinary Ca^{2+} excretion compared to females, a feature accompanied by a reduced renal expression of Ca^{2+} transport proteins. Second, androgen-deficient ORX mice show a significant decline in the urinary excretion of Ca^{2+}, which normalizes after testosterone replacement. Third, similar data were obtained when evaluating the Ca^{2+}/Cr ratio, suggesting that the testosterone-induced increase of urinary Ca^{2+} excretion is due to inhibition of tubular Ca^{2+} reabsorption. Fourth, the mRNA and protein abundance of renal Ca^{2+} transporters was upregulated in ORX mice, whereas the expression of renal Ca^{2+} transporters was suppressed by resupplying these mice with testosterone. Fifth, the serum $1,25(OH)_2D_3$, PTH, and estrogen levels did not differ between the sham-operated, ORX, and ORX + T mice, suggesting that androgens may affect the transcription of the renal Ca^{2+} transporters. Finally, inhibition of transcellular Ca^{2+} transport after DHT treatment was observed in rabbit kidney CNT/CCD primary cell cultures.

Fe(male) sex hormones regulating Ca^{2+} transport

Our observation that male mice have an increased urinary Ca^{2+} excretion than females is in agreement with previous clinical studies evaluating gender differences in humans.[1,2]

ORX induce hypocalciuria in male mice, whereas testosterone supplementation normalized their urinary Ca^{2+} excretion. This was accompanied by a decreased expression of renal Ca^{2+} transport proteins such as TRPV5, NCX1, PMCA1b, and calbindin-D_{28K}. Previous studies evaluating the effect of estrogen on renal Ca^{2+} handling showed that the hormone exerts a direct effect on renal Ca^{2+} reabsorption by upregulation of these Ca^{2+} transport proteins.[3] Thus, both testosterone and estrogen have opposing regulatory properties in terms of renal expression of Ca^{2+} transporters. Similar sex differences have been found for the regulation of the thiazide-sensitive sodium chloride cotransporter (NCC) expressed in the distal convoluted tubules. Chen *et al.*[14] showed that the density of NCC (quantified by [^{3}H]metolazone binding) was twofold higher in female than in male rats. Furthermore, ORX resulted in an increase in metolazone binding sites in males, whereas ovariectomy decreased the binding density in females.[14]

Role of calciotropic hormones in androgen regulation?

In this study serum $1,25(OH)_2D_3$ and PTH did not vary between sham-operated, ORX, and ORX + T mice, suggesting that upregulation of TRPV5 in ORX mice is not mediated by these calciotropic hormones. Conversely, androgens have been previously suggested to affect Ca^{2+} homeostasis by altering the regulation of calciotropic hormones. Some discrepancy has been reported in the literature; in a study by Nyomba *et al.*,[15] the serum concentration of $1,25(OH)_2D_3$ was shown to decrease after ORX in male rats, whereas testosterone replacement therapy restored serum $1,25(OH)_2D_3$ to normal levels. In agreement with our data, a study by Hope *et al.*[16] reported that ORX performed in male rats could not be associated with any changes in active $1,25(OH)_2D_3$ levels. Possible explanations for these discrepancies are currently unclear and may not exclude a contribution of $1,25(OH)_2D_3$ to overall Ca^{2+} handling by androgens.

Here, we showed that the AR is present in TRPV5-expressing cells, which is in line with earlier results showing the presence of the AR in the distal part of the nephron.[17] It is presently unclear whether the activated AR is directly or indirectly involved in decreasing the expression of the investigated Ca^{2+} transporters and hence a higher urinary Ca^{2+} excretion. We investigated this directly by expressing a 5 kb fragment (−5000 to +1) of the mouse TRPV5 promoter coupled to the luciferase gene in androgen-responsive human prostate adenocarcinoma (LnCAP) cells (data not shown). However in these cells, we were not able to observe any effect of DHT on luciferase activity. Currently, it remains unclear how large the promoter fragment should be to adequately drive TRPV5 transcription in response to androgens. In addition, transcriptional regulators that could be necessary for the androgen-mediated inhibition may be absent in this cell system. The exact mechanism whereby testosterone alters TRPV5 expression remains to be clarified.

Short- vs. long-term effects of androgens on Ca^{2+} homeostasis

In this study, we aimed to evaluate the primary effect of androgens on renal Ca^{2+} handling. The inhibitory effect of testosterone on renal Ca^{2+} reabsorption seems at variance with the increased Ca^{2+} excretion found in elderly men with androgen deficiency, which is thought to be associated with male osteoporosis during aging.[18–20] However, the short-term renal effects of androgen deficiency presented here should be separated from the long-term consequences of andropause in terms of bone remodeling. This issue was appropriately addressed in a study of Mauras *et al.*[13] They studied young men who were made hypogonadal for different time periods by injection of a gonadotropin-releasing hormone agonist. The contribution of Ca^{2+} released from bone to urine losses was shown to remain unchanged for 4 weeks, but, thereafter, significantly increased upon 10 weeks after induction of hypogonadism. In our experiments, we studied the effects of androgen deficiency in mice within the time frame of 2 weeks to avoid the possible interference of Ca^{2+} released from bone. We could clearly delineate a change in intrarenal Ca^{2+} transport. Furthermore, we substantiated our *in vivo* results by experiments in isolated rabbit kidney CNT/CCD primary cell cultures. These cells express endogenous TRPV5 and calbindin-D_{28K}, and are a consistent model to investigate active transepithelial Ca^{2+} transport *ex vivo*.[11] Here, we found that incubation with the nonaromatizable androgen DHT (10 nmol/l, which is in line with a physiological concentration of testosterone) for 24 h resulted in a marked inhibition of apical-to-basolateral Ca^{2+} transport. This finding further supports the inhibitory role of androgen on the regulation of renal active Ca^{2+} reabsorption *in vivo*.

In conclusion, this study provides evidence that androgens contribute to sex differences observed in renal Ca^{2+} handling by inhibiting the expression of renal Ca^{2+} transport proteins. Furthermore, this effect is independent of calciotropic hormones or estrogen.

MATERIALS AND METHODS

Animal experiments

Experiment A: male ($n = 15$) and female ($n = 15$) C57BL6 mice, 12 weeks of age, were housed in a light and temperature-controlled room with *ad libitum* access to deionized drinking water and standard chow (0.28% (wt/wt) NaCl, 1.00% (wt/wt) Ca, 0.22% (wt/wt) Mg; LabDiet, Richmond, IN, USA). After acclimatization, mice were housed in couples in metabolic cages and 24 h urine was collected. Animals were killed at the end of the experiment. Blood was colleted and the kidneys dissected out and processed for further analyses.

Experiment B: male C57BL6 mice ($n = 36$), 12 weeks of age, were housed and fed as described for experiment A. After acclimatization, the mice were randomly allocated to either a sham or bilateral ORX operation under (1.5%) halothane anesthesia and divided into three groups ($n = 12$ in each group): (1) sham-operated mice serving as control animals, (2) ORX mice treated with vehicle, and (3) ORX mice treated with Sustanon 250 (ORX + T) subcutaneously (250 mg/kg per week; Sustanon 250 is a long-acting mixture of testosterone ester[21]) (Organon Laboratories, Cambridge, UK).

The operation was performed under halothane anesthesia. After 2 weeks, these mice were housed in couples in metabolic cages and 24 h urine was collected. Thereafter, animals were killed as in experiment A. The animal ethics boards of the National Defense Medical Center (Taipei, Taiwan) approved all animal experimental procedures.

Urine and serum analyses

Urine and serum concentrations of Cr and Ca^{2+} were determined using an automated analyzer (AU 5000 chemistry analyzer; Olympus, Tokyo, Japan). Serum $1,25(OH)_2D_3$ levels were determined by an $[I^{125}]1,25(OH)_2D_3$ RIA assay (DiaSorin, Stillwater, MN, USA). Serum PTH concentrations were determined by IMMULITE PTH assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Both serum testosterone and estrogen concentrations were measured using chemiluminescence immunoassays (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).

Determination of intestinal Ca^{2+} absorption

Male and female wild-type mice (7–8 weeks of age) breed off a C57Bl/6 strain were used as previously described.[22] Radioactive $^{45}Ca^{2+}$ was given by oral gavage, after an overnight fast. Intestinal absorption was determined by repeatedly measuring serum $^{45}Ca^{2+}$ content as described in detail.[22] Briefly, the solution used to measure Ca^{2+} absorption contained 0.1 mmol/l $CaCl_2$, 125 mmol/l NaCl, 17 mmol/l Tris, 1.8 g/l fructose, and 20 μCi $^{45}CaCl_2$ per ml. Blood samples were obtained at 1, 2, 3, 4, and 7 min after oral gavage. Serum $^{45}Ca^{2+}$ content was determined by liquid scintillation counting. Changes in serum Ca^{2+} concentration were calculated from the $^{45}Ca^{2+}$ content of the serum samples and the specific activity of the administrated $^{45}Ca^{2+}$.

Expression of renal Ca^{2+} transporters

To determine mRNA expression levels, we extracted total RNA from kidney using Trizol Total RNA Isolation Reagent (Sigma, St Louis, MO, USA). The obtained total RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 1.5 μg of total RNA was reverse transcribed by Moloney-murine leukemia virus-reverse transcriptase (Promega, Madison, WI, USA), as previously described.[3] The obtained cDNA was used to determine TRPV5, calbindin-D_{28K}, NCX1, and PMCA1b mRNA levels in kidney cortex by real-time quantitative RT-PCR (ABI Prism 7700 Sequence Detection System; PE Biosystems, Rotkreuz, Switzerland). The expression level of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase was used as an internal control to normalize differences in RNA extractions and reverse transcription efficiencies. The primers and fluorescent probes used are as previously described (MdBio, Taipei, Taiwan).[3,23]

For protein expression quantification, total kidney lysates of the mice were prepared and analyzed as previously described.[24] Briefly, proteins in kidney lysates were separated using SDS–polyacrylamide gel electrophoresis and subsequent electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Blots were incubated with rabbit anti-calbindin-D_{28K} (Sigma) or rabbit β-actin (Sigma) polyclonal antibodies. Subsequently, the blots were incubated with a goat anti-rabbit peroxidase-labeled secondary antibody (Sigma). Immunoreactive protein was detected by the enhanced chemiluminescence method (Pierce, Rockford, IL, USA). Protein expression of the immunopositive bands was quantified by the use of pixel density scanning and computed calculation using the Molecular Analyst software of Bio-Rad Laboratories (Hercules, CA, USA).

Immunohistochemical labeling of renal Ca^{2+} transporters

Kidneys were immersion-fixed in 1% (wt/v) periodate-lysine-paraformaldehyde for 2 h at room temperature, and incubated overnight at 4 °C in phosphate-buffered saline containing 15% (wt/v) sucrose. Subsequently, 7 μm sections were cut from liquid nitrogen frozen kidney tissue samples for immunohistochemistry as previously described.[25] For detection of TRPV5 protein abundance, kidney sections were stained with a guinea pig anti-TRPV5 antibody, as described,[25] and a mouse anti-calbindin-D_{28K} antibody (Sigma). TRPV5 and calbindin-D_{28K} were visualized by staining those sections with goat anti-guinea pig and goat anti-mouse Alexa 488-conjugated anti-IgGs (Sigma), respectively. Next, to semiquantify the TRPV5 protein expression, five digital images of each kidney section were taken with a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY, USA) and the integrated optical density was measured by computer analysis with the Image-Pro Plus version 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

Double staining using anti-TRPV5 and a rabbit anti-AR antibody (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using the TSA™ Plus Fluorescein amplification system (PerkinElmer, Groningen, the Netherlands) for TRPV5 and a goat anti-rabbit IgG conjugated to Alexa 594 for visualization of the AR. Confocal pictures were acquired with an Olympus FV1000 laser scanning microscope (Center Valley, PA, USA). Differential interference contrast was superimposed on the fluorescence images.

Primary cultures of rabbit CNT/CCD and determination of transepithelial Ca^{2+} transport

Rabbit kidney CNT and CCD cells were immunodissected from the kidney cortex of New Zealand White rabbits (5 weeks of age) using R2G9 antibodies and set in primary culture on permeable filter supports (0.33 cm^2; Corning-Costar, Cambridge, MA, USA), as previously described in detail.[26] The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Gibco, Paisley, UK) supplemented with 5% (v/v) decomplemented fetal calf serum, 10 μg/ml ciproxin, 10 μl/ml nonessential amino acids, 5 μg/ml insulin, 5 μg/ml transferrin, 50 nmol/l hydrocortisone, 70 ng/ml prostaglandin E_1, 50 nmol/l Na_2SeO_3, 5 pmol/l triiodothyronine, and 5 μmol/l indomethacin. Before incubation with DHT, the transepithelial resistance (R) was measured to assure the integrity of the cells. In all filters used, the R was greater than 400 $\Omega \times cm^2$.

Five days after seeding, cells were incubated for 24 h with 10 nmol/l (5α,17β)-17-hydroxy-androstan-3-one (dihydrotestosterone (DHT); Sigma), or vehicle (ethanol absolute), at the apical and basolateral compartments. Transport assays were performed on confluent monolayers the following day as previously described.[26,27] Briefly, confluent monolayers were washed twice and preincubated in physiological salt solution (140 mmol/l NaCl, 2 mmol/l KCl, 1 mmol/l K_2HPO_4, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 5 mmol/l glucose, 5 mmol/l L-alanine, 5 μmol/l indometacin, and 10 mmol/l HEPES-Tris (pH 7.4)) for 15 min at 37 °C. The cell monolayers were subsequently incubated in physiological salt solution for another 90 min to measure transepithelial Ca^{2+} transport. During the transport assay 10 nmol/l of DHT was added to both the apical and basolateral compartments. At the end of the experimental period, the apical medium was removed and assayed for total Ca^{2+} concentrations, using a colorimetric assay kit (Roche, Mannheim,

Germany). Under these experimental conditions, net apical-to-basolateral Ca^{2+} transport is linear for at least 3 h. Transepithelial Ca^{2+} transport was determined as nmol/h/cm^2.

Statistical analyses

Values are expressed as means ± s.e.m. Statistical significance ($P<0.05$) between groups was determined by an unpaired Student's *t*-test (for comparisons between two individual groups) or by one-way analysis of variance (for multiple comparisons). All analyses were performed using the StatView Statistical Package Software (Power PC, version 4.51; Berkeley, CA, USA).

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