Mapping of sex hormone receptors and their modulators along the nephron of male and female mice

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Abstract

Renal functions are regulated by steroid sex hormones, but the exhaustive identification of their receptors along the nephron is still lacking. Here, we have localized all known nuclear or membrane-bound sex hormone receptors and some of their activators along the nephron of male and female mice. Almost all receptors are present in male and female kidney, some of them having very restricted localization. Only one gene tested among 11 (ARA54) exhibits a gender difference in the level of its expression. This first “renal map” of sex steroid receptor expression may serve as a pre-requisite for investigating the role of these hormones on kidney functions.

1. Introduction

Renal functions are directly affected by gender. Indeed, urinary Na and K excretion are lower in women than in men [1], which could be linked to differences in expression levels of Na and K transporters [2]. A potential explanation for these renal gender differences could be the hormonal status, since sex steroid hormones have been reported to influence many aspects of the renal functions (mainly involved in sodium, calcium and phosphate homeostasis [3]). These global physiological actions are, in part, due to regulation of expression of renal ion transporters [4–6]. All together, these data point out that kidney is the target of all three main steroid sex hormones but the molecular mechanisms, and mainly the identification of the receptors and the part of the nephron involved in these processes are still unknown.

Steroid sex hormones act via binding to specific receptors which may be divided into two categories: (1) the classical nuclear receptors for progesterone (nPR), estradiol (nER) and testosterone (nAR) and (2) membrane-bound receptors like PGMRC1 [7] and mPR [8] for progesterone and GPR30 (also called mER) for estradiol [9]. In addition, it has been shown that the transcriptional activity of nuclear receptors (mainly in the case of nAR) was dependent on the presence of different modulators [10].

Regarding sex hormone nuclear receptors, their presence has been confirmed in whole kidney [11] but, as recently mentioned by Sabolic et al. [11], “their affiliation with defined nephron segment was either not tested or not clearly demonstrated”. Indeed, classical strategies, trying to answer this question, have mainly consisted to use autoradiographic (with tritiated ligands) or immunohistochemical techniques but their results are difficult to reconcile (for examples and references see [11]). Therefore, it appears that autoradiographic studies efficiently localize binding sites but does not allowed a clear identification of the receptors involved in this process. As for immunological approaches, their results are highly dependent on the quality (specificity and sensitivity) of the antibody used. Possible differences between species regarding expression and localization of sex hormone receptors has also to be stressed-out and in some cases could explain some reported discrepancies.

In addition to this uncertainty on the localization of nuclear receptors, there is no data available regarding the potential gender specificity for the expression and the tubular localization of nuclear receptor regulators or of the membrane-bound receptors (excepted for mPR that we recently characterized [12]). Here, we pursue the effort to identify and localize each of these receptors and activators along the nephron of male and female mice.
2. Materials and methods

2.1. Animals and renal tubule isolation

Animal experiments were carried out according to the French legislation. Kidneys from male and female CD1 mice (3 or 10 weeks old, Charles Rivers Breeding Laboratories) were perfused as described previously [12]. The following structures were microdissected according to morphologic and topographic criteria: proximal convoluted tubules (PCT), proximal straight tubules (PST), medullary and cortical thick ascending limb of Henle’s loop (mTAL and cTAL), connecting tubules (CNT), cortical and outer medullary collecting duct (CCD and OMCD).

2.2. RNA extraction, reverse transcription and real-time PCR

RNAs extracted as previously described [13] from pools of 20–30 microdissected tubules were reverse-transcribed (Roche Diagnostics, France) according to the manufacturer’s instructions. Real-time PCRs were performed on a LightCycler (Roche Diagnostics) with the 480 SYBR green I Master kit (Roche Diagnostics, France) according to the manufacturer’s instructions except that the total reaction volume was reduced 2.5-fold. PCRs were performed with cDNA quantity corresponding to 0.1 mm of tubules. No DNA was detectable in samples that did not undergo reverse transcription or in blank run without cDNA. Cyclophilin expression does not vary detectable in samples that did not undergo reverse transcription and what are their localization along the nephron. Genes for which a pre-requisite to understand the effects of sex hormones on renal functions, is to determine which types of receptors are present in the kidney and what are their localization along the nephron (see Fig. 1A for schematic representation). To achieve this goal, we have chosen to develop an approach based on the mRNA quantification by QPCR on manually microdissected renal tubules from male and female mice. This strategy should avoid the drawbacks encountered in previous studies using immunological or autoradiographic techniques.

As shown in Fig. 1B and C, progesterone receptors (nPР and PGMRC1) transcripts are both present in the distal part of the nephron from the CNT to the OMCD. However, only PGMRC1 is expressed in the proximal structures (PCT and PST). Thick ascending limb expresses PGMRC1 and no nPR. In addition, the restricted expression of mPRx to proximal tubules and the presence of mPRy all along the nephron [12] indicate that progesterone may have a broad action on renal functions by acting specifically on one or another part containing CNT, CCD and OMCD (see Section 2).

2.3. Protein homogenate and Western blot

After sacrifice, kidneys were removed, weighed and cut into small pieces. Minced tissues were then homogenized in an isolation buffer (250 mM sucrose, 1 mM EDTA and 10 mM NaOH–HEPES, pH 7.4) containing a protease inhibitor cocktail (Roche) with a Dounce homogenizer (10 passes). For isolation of the post-nuclear supernatant, the homogenate was centrifuged at 10000 g for 10 min. Protein contents were determined using the BCA protein assay (Pierce). Thirty micrograms of proteins was denaturated by 2X protein sample buffer (4.8% SDS, 6.9% sucrose, 0.012% bromophenol blue, 2.1% -mercaptoethanol) and heated 3 min at 95 °C. The samples were then resolved onto a 10% SDS–polyacrylamide gel. Western blot experiments were performed according the standard procedure using anti-ARA54 antibodies (AbCam, ab56605).

2.4. Statistical analysis

Our data were analyzed by a Student t-test for potential differences between gender, for the same type of segments (one asterisk, P < 0.05). Our data were then studied independently of the gender, by an ANOVA test of variance to identify genes having a variable expression along the nephron. Genes for which a P value were <0.05, were then analyzed further. For clarity, we grouped data from segments of the same part of the nephron (proximal part containing PCT and PST; Henle’s loop containing cTAL and mTAL and distal part containing CNT, CCD and OMCD) being established that inside these groups, the gene expression is not statistically different. We have, then, compared these three different groups by a Student t-test (one sharp symbol, P < 0.05; two sharp symbols, P < 0.01).

3. Results and discussions

Expression of progesterone receptors along the male and female nephron. Schematic representation of a nephron. Real-time PCR was performed using specific primers for nPR (B) or PGMRC1 (C) from male (black bars) or female (white bars) mouse renal segments (PCT: proximal convoluted tubule, PST: proximal straight tubule, m and cTAL: medullary and cortical thick ascending limb of Henle’s loop, CNT: connecting tubule, CCD: cortical collecting duct, OMCD: outer medullary collecting duct). Gene encoding for cyclophilin was used to standardize the quantity of cDNA in each sample. ANOVA tests were performed (SigmaStat 3.5) for both proximal part containing CNT, CCD and OMCD (see Section 2).
other receptor. These results confirm that progesterone may have a specific action on renal function independently of its putative agonist or antagonist effects on mineralo- and glucocorticoid receptors.

As for estrogen receptors, there is no nERβ mRNA expression either in male or in female mouse kidney. The nERα isoform was mainly expressed in proximal tubules (PCT and PST, see Fig. 2A) in both genders and to a lower level in distal segments (CNT, CCD and OMCD). These results fit well with the description of abundant estrogen binding sites in cytoplasm or nucleus of proximal tubule cells whereas only few cells in distal tubule were labeled [14]. In addition to the classical nuclear estrogen receptor, a membrane-bound receptor (GPR30 or mER) has been recently identified. The expression profile of this transcript is shown in Fig. 2B. Conversely to nER, mER is more abundant in the distal part of the nephron than in proximal tubules, in both genders. This dichotomy may suggest more complicated renal actions of estrogens than previously expected with a genomic action regulating the expression of several transporters (almost exclusively located in proximal tubules, reviewed in [11]) and a rapid action specifically mediated in distal tubules by the mER. Interestingly, mER knock-out mice have been generated recently [15,16]. Their renal phenotype has not been studied but these mice exhibit a higher blood pressure than their littermate [16]. In view of the specific localization of mER that we showed here, it would be interesting to investigate the possible link between mER and Na transport and transporters in the distal nephron.

Up to now, only one androgen receptor has been identified, corresponding to the nuclear androgen receptor nAR. Our data

Fig. 2. Expression of estrogen receptors along the male and female nephron. Real-time PCR was performed using specific primers for nERα isoform (A) and for mER (B) on the same sample from male (black bars) and female (white bars) as described in Fig. 1. Results are mean ± S.E. of four different male or female mice. ANOVA tests were performed for both genders and showed that nER and mER expression are significantly different along the nephron (* P < 0.05 in males and females). Groups of segments representing specific part of the nephron have been considered for the clarity of the comparison (see Section 2).

Fig. 3. Expression of androgen receptor (nAR) and androgen receptor activators (ARAs) along the male and female nephron. Real-time PCR was performed using specific primers for nAR (A), ARA54 (B), ARA55 (C), ARA70 (D) and ARA267 (E) on the same samples from male (black bars) and female (white bars) described in Fig. 1. Results are mean ± S.E. of four different male or female mice. (F) ARA54 expression has been investigated with an anti-ARA54 antibody. Western blot analysis was performed using kidney homogenates (30 μg of protein) from male (10 weeks old, lanes 1–3; 3 weeks old, lanes 7–9) or female (10 weeks old, lanes 4–6; 3 weeks old, lanes 10–12). ANOVA tests were performed for both genders and showed that nAR, but not ARA54, ARA55, ARA70 or ARA267, expression are significantly different along the nephron (P < 0.01). Groups of segments representing specific part of the nephron have been considered for the clarity of the comparison (see Section 2).
(Fig. 3A) clearly indicated a restricted localization of this receptor in the proximal tubules of male and female mice as we did not observe any nAR transcript in distal segments. Previous studies [14,17] in mouse or rat have, also, shown predominant localization of nAR in proximal tubules, excepted in one [18]. In contrast to our data, these studies have also reported possible expression of nAR in more distal tubules where a weak signal (compared to proximal tubules) was observed in few cells. We can not explain whether this apparent discrepancy is due to differences in species or to immunohistochemical artifact, but the absence of mRNA in mouse CNT, CCD and OMCD strongly supports the absence of the protein. A testosterone binding site [14] in the segments of distal nephron may also suggest the presence of still not characterized androgen receptors.

Surprisingly, the different co-activators of the androgen receptor (Fig. 3B–E) do not exhibit the same restricted localization than the nAR. A possible explanation for this apparent discrepancy is that these proteins have multiple functions. For instance, they have been shown, in vitro, to modulate the activity of either estrogen (ARA70 and ARA267, [19,20]) or progesterone nuclear receptors (ARA54 and ARA55 [21,22]). Moreover, all four androgen receptor activators tested in this study exhibit molecular and cellular functions not directly related to steroid nuclear receptors (ubiquitin ligase, protein adapter, histone methyl transferase, for review see [20]). These other functions may be required in all renal segments independently of the presence of nAR. A striking observation is that expression of ARA54 is lower in male mice than in female (Fig. 3C).

We confirmed this observation by analyzing the protein expression of ARA54 on protein extracts from male or female kidneys. As shown in Fig. 3F, a mouse anti-ARA54 recognized two bands, one (a doublet) with an apparent molecular weight of 66–69 kDa and another migrating faster at 54 kDa that we have considered as another migrating faster at 54 kDa that we have considered as (a doublet) with an apparent molecular weight of 66–69 kDa and another migrating faster at 54 kDa that we have considered as (a doublet) with an apparent molecular weight of 66–69 kDa and another migrating faster at 54 kDa that we have considered as (a doublet) with an apparent molecular weight of 66–69 kDa and another migrating faster at 54 kDa that we have considered as. The 54 kDa-band is more abundant in both adult (10 weeks old, lanes 4–6) and young (3 weeks old, lanes 10–12) female mice than in adult (lanes 1–3) or young (lanes 7–9) male mice, indicating that ARA54 expression does not depend on the sexual maturity of the animals. Further analysis is required to understand the consequences of such difference on renal functions in male and female.

To study the global relationship between gene expression, gender and tubular localization, we performed a hierarchical, unsupervised clustering analysis from our QPCR data. We included in this analysis the expression profile for mPRx and mPRy that we published previously [12]. As shown in Fig. 4, there is no cluzterization according to hormone specificity or to gender. The three clusters only emerging reveal a segregation depending exclusively on the nature of the nephron segment. This analysis reveals that the three classes of hormones may affect the function of proximal segments by acting either on classical nuclear receptors (nAR and nERx) or on membrane-bound receptors (mPRx). Activation of these receptors could affect very specific functions of proximal tubules such as renal phosphate reabsorption. Distal tubules, on the other hand, specifically expressed mER and nPR which may suggest their involvement in the fine tuning of sodium and potassium homeostasis.

All together, our results showed that mice kidney is not gendered. It is ready to cope with any specific physiological demands, linked to gender or reproductive states (puberty, ovarian cycles, pregnancy, etc.) without having to adapt its responding “equipment” to hormonal variations. Finally, the origin of the sexual dimorphism of the renal functions cannot be explained by a potential difference in the expression of sex steroid receptors between male and female kidney.

Acknowledgments

We thank Lydie Cheval and Alain Doucet from the UPMC/CNRS ERL7226 for technical assistance and fruitful discussions. This study was supported by grants from the INSERM program for Nephrology and Urology PNRNU0701 (G.C.) and by the CNRS program ATIP (G.C.).

References


