Aldosterone postnatally, but not at birth, is required for optimal induction of renal mineralocorticoid receptor expression and sodium reabsorption.

Laetitia Martinerie, Say Viengchareun, Géri Meduri, Hyung-Suk Kim, James Luther, Marc Lombes

Aldosterone postnatally, but not at birth, is required for optimal induction of renal mineralocorticoid receptor expression and sodium reabsorption

Abbreviated Title: Aldosterone regulation of renal MR expression

Laetitia Martinerie[1,2,6], Say Viengchareun[1,2], Geri Meduri[1,3], Hyung-Suk Kim[4], James M. Luther[5], Marc Lombès[1,2,6,7]

[1] *Inserm, U693, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France;*

[2] *Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin Bicêtre, F-94276, France;*

[3] *Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service de Génétique moléculaire, Pharmacogénétique et Hormonologie, Le Kremlin Bicêtre, F-94275, France;*

[4] *Department of Pathology, University of North Carolina, Chapel Hill, NC, USA;*

[5] *Division of Clinical Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA;*

[6] *Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin Bicêtre, F-94275, France;*

[7] *PremUp Foundation, Paris, F-75006, France.*

*** Corresponding author:**

Dr Marc LOMBES, MD, PhD
Inserm U693, Faculté de Médecine Paris-Sud
63, rue Gabriel Péri, 94276 Le Kremlin Bicêtre Cedex France
Tel 33 1 49 59 67 09
Fax 33 1 49 59 67 32
Email: marc.lombes@u-psud.fr

Reprint request should be addressed to Dr Marc LOMBES

Key words: Newborns, aldosterone-synthase, aldosterone, mineralocorticoid receptor, kidney

Statement and financial support: The authors have nothing to disclose.

Precis: Aldosterone is dispensable at birth but rather crucial in the postnatal period for optimal induction of the mineralocorticoid signaling pathway and for sodium homeostasis.

Word count of abstract: 248

Word count: **3897**

Abstract

Sodium wasting during the neonatal period is the consequence of a physiological aldosterone resistance, related to a low renal mineralocorticoid receptor (MR) expression at birth, both in humans and mice. To investigate whether aldosterone is involved in the neonatal regulation of MR expression, we compared aldosterone and corticosterone levels and renal MR expression by quantitative real time PCR, between aldosterone-synthase knock-out (ASKO), heterozygous and wild type (WT) mice, at birth (D0) and eight days postnatal (D8). Analysis of MR transcripts showed a similar expression profile in all genotypes, demonstrating that the lack of aldosterone does not modify either the low renal MR expression at birth or its postnatal induction. However, mRNA levels of the α subunit of the epithelial sodium channel (ENaC), a MR target gene, were significantly higher in WT compared to ASKO mice, both at D0 and D8, despite high corticosterone levels in ASKO mice, indicating that aldosterone is required for optimal renal induction of ENaC. Using organotypic cultures of newborn WT kidneys, we confirmed that aldosterone does not regulate MR expression at birth, but is instead capable of increasing MR expression in mature kidneys, unlike dexamethasone. In sum, we demonstrate both *in vivo* and *in vitro*, that, whereas aldosterone has no significant impact on renal MR expression at birth, it is crucial for optimal MR regulation in postnatal kidneys and for appropriate hydroelectrolytic balance. Understanding of MR regulatory mechanisms could therefore lead to new therapeutic strategies for the management of sodium loss in preterms and neonates.

Introduction

In the neonatal period, the human kidney displays a tubular immaturity, with associated sodium wasting, negative sodium balance, and impaired water reabsorption (1). This inability to maintain homeostatic function is accentuated in preterm infants (2-4) and represents a critical problem that pediatricians have to deal with. Therefore, a better understanding of water and sodium regulation during this specific developmental period is of major importance in order to propose new therapeutic strategies for the management of preterm infants.

Renal sodium reabsorption is mainly controlled by aldosterone, a steroid hormone synthesized in the adrenal gland zona glomerulosa, secondary to renin stimulation *via* angiotensin II and to potassium stimulation (5). In the distal nephron, aldosterone, by binding to its receptor, the mineralocorticoid receptor (MR), a transcription factor (6), tightly regulates the expression and activity of several transporting proteins implicated in sodium homeostasis, including the alpha subunit of the epithelial sodium channel (αENaC) (7). The selectivity of the mineralocorticoid signaling pathway in the epithelial cells is controlled at a prereceptor level by the 11β-hydroxysteroid dehydrogenase type 2 enzyme (11βHSD2) which metabolizes cortisol (or corticosterone in rodents) into inactive compounds, uncapable of MR binding (8, 9). We have previously demonstrated that the neonatal sodium wasting is related to a physiological transient renal aldosterone resistance (10). We have also established that this physiological aldosterone resistance is associated in both mice and humans with a low renal MR expression at birth, both at the mRNA and protein level (11). This low renal MR expression in newborns is followed by a significant increase in the postnatal period, with a complete renal tubular expression developed at 8 postnatal days in mice and during the first year of life in humans, paralleling renal maturation. The underlying mechanisms, responsible for the low renal MR expression at birth and its postnatal increase, are currently unknown. Since aldosterone levels are very high at birth and have a tendency to decrease in the postnatal period, mirroring MR expression (11), we hypothesized that these high hormonal levels could contribute to the low neonatal renal MR expression. To investigate the role of aldosterone on neonatal MR regulation, we utilized the aldosterone-synthase knock-out mouse model ($AS^{-/-}$). These transgenic mice were originally generated

by Hyung-Suk Kim, using standard gene targeting methods, with a final construct lacking exons 1, 2, part of exon 3 and introns 1 and 2 of the AS gene (12), and have undetectable levels of plasma aldosterone. $AS^{-/-}$ genotype is compatible with fetal development but newborns fail to thrive postnatally and about 30% die between day 7 and 28 (12). Adult $AS^{-/-}$ mice are small, weigh 25% less than wild type animals, have low blood pressure, abnormal electrolyte homeostasis (increased plasma concentrations of K^+, Ca^{2+} and Mg^{2+}, decreased concentrations of $HCO3^-$ and Cl^- but no difference of plasma Na^+ level under normal diet) and altered water metabolism (higher urine output, decreased urine osmolality, and impaired urine concentrating and diluting ability). Higher levels of plasma corticosterone and strong activation of the renin-angiotensin system are observed. In contrast, $AS^{+/-}$ mice have normal plasma aldosterone concentrations, normal blood pressure and no electrolyte disturbances (13). To evaluate whether renal MR expression in the neonatal period is dependent on aldosterone, we have quantified and compared MR mRNA steady state levels in the kidneys of $AS^{-/-}$, $AS^{+/-}$ and wild type littermates, using quantitative real-time PCR. To determine whether maternally-derived aldosterone exposure *in utero* modifies MR expression at birth, we conducted two breeding strategies, utilizing pups derived from either $AS^{-/-}$ or $AS^{+/-}$ mice. We have also analyzed the expression of other actors of the mineralocorticoid (11βHSD2, αENaC) and glucocorticoid (GR) signaling pathways, and measured hormonal status. Our results were subsequently confirmed *in vitro*, using organotypic cultures of wild type newborn murine kidneys. We demonstrate that, whereas aldosterone is dispensable for MR regulation at birth, it is pivotal for optimal induction of renal MR expression and sodium homeostasis in the postnatal mature kidney, effects that cannot be compensated by the glucocorticoid signaling pathway.

Materials and Methods

Animals and Breeding strategy

All experiments were reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee. $AS^{-/-}$ mice, previously described (12), were generated on a 129 background and backcrossed over ≥15 generations onto the C57Bl6/J strain (Jackson Laboratory, Bar Harbor, ME). Mice were genotyped by real-time PCR (Applied Biosystems 7900HT, Foster City, CA) using Taqman probes for a sequence in the *Cyp11b2* gene and for a portion of the gene contained in the neomycin resistance cassette, as previously described (14). Animals were housed in a temperature-controlled facility with a 12-hour light/dark cycle. $AS^{-/-}$ female mice were mated with $AS^{-/-}$ male mice in order to obtain $AS^{-/-}$ littermates. $AS^{+/-}$ female mice were mated with $AS^{+/-}$ male mice in order to obtain $AS^{-/-}$, $AS^{+/-}$ and wild type littermates. Mice were sacrificed at birth and at post-natal day 8 by decapitation. Kidneys were immediately collected, snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Hormonal Analyses

Trunk blood was collected into dipotassium-EDTA tubes (Microvette CB K2E, Sarstedt AG & Co), centrifuged at 6,000 rpm for 5 min, and plasma was stored at -80°C until assay. Aldosterone was determined as previously described (14) using a radioimmunoassay utilizing ^{125}I-aldosterone (MP Biomedicals, Irvine CA), a primary antibody to aldosterone (NIDDK National Hormone & Peptide Program, Torrance CA), and a secondary anti-rabbit gamma globulin antibody (Linco Research, St. Charles, MO). Corticosterone was measured using a commercially available radioimmunoassay kit (ImmuChem Double Antibody Corticosterone Kit, MP Biomedicals, Irvine, CA) as described in (14).

Organotypic cultures

Kidneys were collected from wild type mice on the day of birth (D0) or at postnatal day 8 (D8). Each D0 kidney was incubated for 15 min in 150 µl of accutase (PAA Laboratories, Les Mureaux, France) and D8 kidneys were incubated for 1 h in 300 µl of trypsin (Invitrogen, Cergy-Pontoise, France).

Then, kidneys were manually dissected with a needle. Homogenates were pooled and centrifuged for 3 min at 300 g, supernatant was withdrawn and cellular pellet was resuspended in a specific epithelial medium described below. Cellular suspensions were then seeded on collagen I-coated 12-well plates (Collagen I from Institut Jacques Boy, France), and routinely cultured for seven days at 37°C in a humidified incubator gassed with 5% CO2 within an epithelial medium composed of DMEM/HAM's F12 (1:1), 2 mM glutamine, 50 nM dexamethasone, 50 nM sodium selenite, 5 µg/ml transferrin, 5 µg/ml insulin, 10 ng/ml EGF, 2 nM T3, 100 U/ml penicillin/streptomycin, 20 mM HEPES, pH 7.4, 5% dextran charcoal-treated serum and 1% amphotericin B (the latter added to the medium for the first 48 h of culture only).

To investigate aldosterone and corticosteroid action, the epithelial medium was replaced at day 7 of culture by a minimal medium (MM), which has the same composition as the epithelial medium but lacks dexamethasone and dextran charcoal-treated serum. After 24 h in MM, either ethanol or aldosterone or dexamethasone, as well as spironolactone, were added to the medium for 24 h as indicated.

RT-PCR and Quantitative real-time PCR

Total RNA was extracted from tissues or cells with the TRIZOL reagent (Invitrogen) according to the manufacturer's recommendations, and RNA was thereafter processed for RT-PCR, as previously described (11). One µg of total RNA, isolated from frozen samples, was subjected to DNase I Amplification Grade treatment (Invitrogen) and then reverse-transcribed by use of High-Capacity cDNA RT kit from Applied Biosystems (Courtaboeuf, France). Samples were diluted 10-fold then 1/20 of the reverse transcription reaction was used for qRT-PCR using the Fast SYBR® Green Master Mix (ABI, Applied Biosystems) containing 300 nM of specific primers (supplemental Table). qPCR was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Reaction parameters were as follows: 95°C for 20 s, then 40 cycles at 95°C for 1 s and 60°C for 20 s. For standards preparation, amplicons were purified from agarose gel and subcloned into pGEMT-easy plasmid (Promega, Charbonnières-les-Bains, France) and sequenced to confirm the identity of each sequence. Standard curves were generated using serial dilutions of linearized standard plasmids,

spanning 6 orders of magnitude. Standard and sample values were amplified in duplicate and analyzed from three independent experiments. Ribosomal 18S was used as an internal control for data normalization. Relative expression of a given gene is expressed as the ratio attomoles of specific gene/femtomoles of 18S. Results are mean ± SEM and represent the relative expression compared with that obtained in $AS^{-/-}$ mice kidneys at D0, which was arbitrarily set at 1. Supplemental Table indicates primer sequences of genes analyzed by qRT-PCR.

Immunocytochemistry

Cells were fixed in 10% buffered-formol in PBS (pH 7.3) for 15 min and then washed 3 times in PBS before processing for immunocytochemistry, as previously described (11). For immunocytochemical analyses, we used the monoclonal anti-MR antibody clone 6G1, generously provided by Dr Gomez-Sanchez (University of Mississippi, USA) (15), at the dilution of 1:40.

Statistical analyses

Results are expressed as mean ± SEM of at least three independent analyses with at least 6 samples for each developmental stage. Statistical analyses were performed using a non parametric Mann Whitney test (Prism4, Graphpad Software, Inc., San Diego, CA), with significant threshold at 0.05.

Results

Breeding strategy

We conducted a specific breeding strategy in order to obtain four different newborn mice with various genotypes and distinct developmental endocrine patterns. We obtained $AS^{-/-}$ pups that had never been exposed to aldosterone, by mating female $AS^{-/-}$ mice with $AS^{-/-}$ male mice. We obtained $AS^{-/-}$ pups that had been exposed to maternal aldosterone, through transplacental crossing (16), by mating $AS^{+/-}$ female mice with $AS^{+/-}$ male mice, which also generated $AS^{+/-}$ littermates and wild type newborn mice. No difference was observed between genotypes regarding weight at birth (1.23 ± 0.07, 1.39 ± 0.02, 1.38 ± 0.04, 1.42 ± 0.11 g, respectively), and at postnatal day eight (3.51 ± 0.23, 3.54 ± 0.19, 4.11 ± 0.17, 4.02 ± 0.27 g, respectively). Kidneys were collected at birth (D0) and at postnatal-day eight (D8). No renal structural abnormality was observed.

Aldosterone and corticosterone levels

Aldosterone and corticosterone levels were measured in $AS^{-/-}$ and wild type (WT) newborn mice at the time of kidney retrieval (Fig 1). As expected, aldosterone was undetectable in $AS^{-/-}$ mice both at D0 and D8. In wild type mice, mean aldosterone level at birth was 466 ± 245 pg/ml and decreased at D8 (94 ± 38 pg/ml) although the difference did not reach significance (p=0.18). Interestingly, we found, in $AS^{-/-}$ animals both at birth and at D8, very high levels of corticosterone (374 ± 317 and 681 ± 376 ng/ml, respectively) were detected in comparison to wild type mice of the same developmental stage, particularly at D8 (p=0.04). Analogous high levels of corticosterone have also been reported in $AS^{-/-}$ adult mice on normal sodium diet (13). One could hypothesize that these high corticosterone levels are secreted to compensate for aldosterone deficiency.

Mineralocorticoid receptor expression

MR transcript levels were measured by quantitative RT-PCR in the kidneys of all four groups of animals at D0 and D8 (Fig 2). No difference was evident at birth between genotypes, therefore suggesting that the low renal MR expression during the neonatal period is independent of aldosterone exposure. In all conditions, a significant increase in renal MR mRNA expression level was observed in

all animals at D8, demonstrating that aldosterone does not intervene into the postnatal upregulation of renal MR expression. However, we found a significant difference ($p<0.05$) in D8 state levels of MR transcripts between $AS^{-/-}$ and WT mice, suggesting that aldosterone may regulate MR expression only in postnatal developed kidneys, when complete tubular maturation is achieved.

11βHSD2 and αENaC expression

We have previously demonstrated in WT mice (11), a parallel evolution throughout renal development between MR, and both the 11βHSD2 enzyme, which confers mineralocorticoid selectivity, and αENaC, a prototypal MR target gene. Since corticosterone levels were very high in $AS^{-/-}$ mice, we wondered whether the expression profiles of 11βHSD2 and αENaC would be modified in the four different genotypes. It has indeed been suggested that the glucocorticoid pathway could be implicated in the regulation of these two genes (17-19). The question raised was whether corticosterone could compensate for aldosterone deficiency in $AS^{-/-}$ mice by inducing αENaC expression *via* binding to the glucocorticoid receptor (GR). The latter is a transcription factor closely related to MR and also known to activate gene expression implicated in sodium transport (18, 19). 11βHSD2 and αENaC mRNA expression were therefore analyzed at D0 and D8 in all genotypes. 11βHSD2 displayed an identical expression profile to that of the MR, with a low expression at birth and a significant increase in the postnatal period, independent of aldosterone status (Fig 3A). On the contrary, αENaC mRNA expression profile appeared strikingly different (Fig 3B). Indeed, αENaC mRNA levels were very low in $AS^{-/-}$ mice both at D0 and D8, with no significant postnatal increase. αENaC expression evolved in parallel with aldosterone levels, with a 1.5-fold and a 2-fold increase at birth in $AS^{+/-}$ and WT mice, respectively, and a 2.5-fold and 3-fold induction at D8 in these same genotypes, compared to levels in $AS^{-/-}$ mice at D0. Of interest, at D0, levels of αENaC transcripts in $AS^{-/-}$ newborns, originating from $AS^{+/-}$ x $AS^{+/-}$ mice, and thus prenatally exposed to aldosterone, were significantly higher than in non-exposed $AS^{-/-}$ x $AS^{-/-}$ littermates, whereas αENaC mRNA levels were identical at D8 in these animals. These results strongly suggest that maternally biosynthesized aldosterone may have a significant impact on fetal kidney αENaC expression. Indeed, at D8, when circulating aldosterone is no longer

detectable in $AS^{-/-}$ mice born from $AS^{+/-}$ mothers, αENaC mRNA levels do not exhibit postnatal induction as happens in $AS^{+/-}$ and WT animals. Therefore, it appears that aldosterone is instrumental for optimal renal induction of αENaC and subsequently for sodium tubular reabsorption in the neonatal period.

Furthermore, it becomes evident that despite the high levels of corticosterone observed in $AS^{-/-}$ mice and their substantial levels of GR expression (see Supplemental Figure), glucocorticoid signaling cannot functionally compensate the lack of mineralocorticoid signaling.

Organotypic cultures

We next decided to establish a reliable cell-based model to further investigate the hormonal regulatory mechanisms of renal MR expression in the neonatal period. We thus chose to grow organotypic cultures of WT newborn (OCD0) and postnatal-day eight (OCD8) mouse kidneys. As presented in Fig 4A, after manual kidney dissection, the nephronic tubules and glomeruli can be clearly visualized in the epithelial medium. Thereafter, epithelial-like tubular cells start to proliferate from nephronic fragments attached to the collagen I-coated plates. After seven days of culture, a full layer of renal tubular cells is observed with the typical features of epithelial cells, most notably the capacity to form domes. Moreover, as shown in Fig 4B, we were unable to immunodetect MR protein in OCD0 after 7 days of culture, while a specific immunolabeling was readily detected in the epithelial-like tubular cells in OCD8, just like that observed in wild type kidneys (11). The cell immunolabelling was intracytoplasmic in the absence of aldosterone (control), or in the presence of a MR antagonist, spironolactone (A-7 + Spiro-6), whereas aldosterone alone (A-7) induced a clear nuclear translocation of the activated MR. These results suggest that primary cultures of renal cells maintain the capacity to express MR protein, and therefore constitute a valuable and reliable experimental cell-based model to investigate the impact of aldosterone (or corticosteroid hormone) exposure on neonatal (D0 and D8) control of renal MR expression.

Impact of Aldosterone on gene expression in organotypic cultures

To explore the impact of mineralocorticoid and glucocorticoid hormones on neonatal renal mRNA expression, we incubated organotypic cultures of wild type D0 and D8 mouse kidneys with various steroids (aldosterone or dexamethasone). No variation in MR mRNA levels was observed in OCD0 after 24 h treatment with either hormone (Fig 4C), providing additional support for the lack of corticosteroid hormone effects on MR expression when comparing D0 $AS^{-/-}$ and WT mice (see Fig 2). However, our cell model was sensitive to glucocorticoid but not aldosterone action since dexamethasone, a steroid compound that is not submitted to metabolic conversion by the 11βHSD2 enzyme (20), was able to induce αENaC expression (data not shown), most likely through glucocorticoid receptor activation. Indeed, the absence of MR protein in OCD0 explains why aldosterone is inefficient in inducing αENaC expression. Finally, in sharp contrast, in OCD8 cultures, clearly expressing MR protein, aldosterone, but not dexamethasone, was able to induce a significant 2-fold increase of MR transcripts ($p < 0.01$) (Fig 4C). This result corroborates the *in vivo* findings obtained in mouse models, revealing a significantly higher MR mRNA expression in WT than in $AS^{-/-}$ mice, particularly at D8.

Discussion

In the present paper, we demonstrate both *in vivo* and *in vitro*, that, at birth, aldosterone has no impact on the renal expression of its specific MR receptor. Physiologically, renal MR expression is extremely low during the neonatal period both in mice and humans, and increases drastically during the postnatal period, in parallel with other actors of the mineralocorticoid pathway such as 11βHSD2, which confers mineralocorticoid selectivity, and αENaC, a prototypal MR target gene required for apical sodium entry and transepithelial reabsorption (11). Herein, we show that the lack of aldosterone biosynthesis observed in $AS^{-/-}$ mice does not modify MR expression profile at this specific period of development, characterized by a persistent low expression at birth and a postnatal induction at D8. Similarly, aldosterone withdrawal or administration does not induce MR mRNA and protein expression in organotypic cultures of newborn kidneys collected on the day of birth.

Interestingly, unlike the MR and 11βHSD2 mRNA expression profiles, which were independent of aldosterone status, αENaC mRNA levels positively correlated with the degree of aldosterone exposure, following the sequence $AS^{-/-} < AS^{+/-} <$ WT. Indeed, in the absence of aldosterone (in $AS^{-/-}$ newborns from $AS^{-/-}$ mothers, and in $AS^{-/-}$ eight-day-old mice), αENaC transcripts levels were low at birth and did not increase postnatally.

The low yet detectable αENaC mRNA levels measured at birth in AS-/- mice, in the absence of aldosterone and concomitant with a very low MR expression, emphasize the redundancy of gene expression regulation. Indeed, the mineralocorticoid pathway is not the sole regulator of αENaC expression. It can also be controlled by other transcription factors, such as the GR (21). We have previously demonstrated that GR is expressed in the neonatal kidney both at the mRNA and protein levels, and is detected in the nuclei of the cortical collecting duct cells, consistent with a functional GR-mediated signaling pathway (11). GR is also detected in the kidney of $AS^{-/-}$ newborns. Therefore the glucocorticoid signaling pathway could account for these detectable αENaC mRNA levels at birth.

However, in comparison, the abundance of αENaC mRNA presented a strict positive relationship with aldosterone status, with a significant increase in αENaC mRNA levels in AS heterozygous and WT newborns as compared to $AS^{-/-}$ mice at D0, associated with a further increase at D8, as previously

demonstrated (11). Moreover, $AS^{-/-}$ mice born from aldosterone-synthase heterozygous mothers and thus exposed to aldosterone during fetal life, presented with higher αENaC mRNA levels at birth compared to $AS^{-/-}$ newborns from $AS^{-/-}$ mothers. In addition, ENaC transcript levels in the kidneys of the former animals decreased during the postnatal period along with the clearance of maternal aldosterone. These results confirm the major role of aldosterone in sodium homeostasis during the neonatal period, which cannot be compensated by the activation of glucocorticoid signaling pathway, despite the high corticosterone levels measured in $AS^{-/-}$ newborn mice and the substantial GR expression. This lack of compensation could also be the consequence of variability in GR potency at this specific period of development (22). These findings are in accordance with Lee *et al.* who have reported the death of one third of $AS^{-/-}$ mice between 7 and 28 days (corresponding to the period of complete kidney maturation and full renal MR expression), from dehydration and failure to thrive (12). The surviving mice presented with hypotension, hyperkalemia and difficulty to concentrate urine, exacerbated under sodium-restricted diet (23), even in presence of high corticosterone levels and subnormal renal GR expression (12, 13). This is reminiscent of MR knock-out newborns which present similar symptoms and die of dehydration at 8 postnatal days (24). Both transgenic mouse models can be rescued by sodium supplementation in the neonatal period (13, 25). These observations are also reminiscent of human infants carrying heterozygous inactivating MR mutations (26) or with aldosterone-synthase deficiency (27-29), who present with failure to thrive and inability to reabsorb sodium in the postnatal period, thus requiring sodium supplementation to survive. Therefore, both aldosterone and MR appear critical for sodium reabsorption and maintenance of sodium and water homeostasis in the postnatal period, and cannot be compensated by a functional glucocorticoid signaling pathway during this specific period of renal development.

It seems particularly interesting that, all the components of the renal mineralocorticoid signaling pathway (MR, 11βHSD2 and αENaC) are physiologically downregulated during the first days of life, both in mouse and human newborns. Therefore, these mechanisms concur to impair sodium reabsorption and facilitate sodium wasting, this physiological condition being of particular importance during these first days, regardless of aldosterone status. This dissociation between aldosterone and mineralocorticoid signaling pathway activation could constitute a renal protection mechanism

preventing inappropriate sodium retention. Alternatively, high physiological aldosterone concentrations could potentially be required for rapid non-genomic effects, as previously suggested (30-32). Nevertheless, these observations could explain why deficiency of either aldosterone biosynthesis or MR expression becomes critical after the first week of life only, both in mice and humans.

Interestingly, at variance with D0, we demonstrated that renal MR expression becomes regulated by aldosterone at D8, most likely once MR mRNA and protein amounts reach threshold levels in mature kidneys. Indeed, we observed a significant increase of MR mRNA levels at D8 between $AS^{-/-}$ and WT mice. These results were corroborated by organotypic culture experiments conducted on D8 WT kidneys, where MR mRNA levels were stimulated by aldosterone but not by dexamethasone. Unlike D0, MR protein expression is readily detected in WT kidneys at D8 and is maintained after one week of organotypic culture. Therefore these results could be consistent with a positive auto-regulatory process. Other authors have already suggested an aldosterone-dependent MR auto-regulation (33, 34). The implication of MR in mediating these aldosterone regulatory effects, was established by using the MR antagonist spironolactone, (33), or by knocking down MR by small interfering RNA (34). Whether this effect is mediated by direct binding of activated MR on the regulatory region of the *MR* gene or involves intermediate transcription factors, like Sp-1 or AP-2 as suggested by Zennaro *et al*. (33), remains to be further investigated. We therefore propose that aldosterone contributes to MR regulation through a positive homologous regulatory loop, most likely *via* MR. This auto-regulation of renal MR expression is not functional at birth owing to the lack or very low abundance of MR protein in the newborn kidneys.

Finally, our results emphasize the multiple mechanisms (in both the mineralocorticoid and glucocorticoid signaling pathways), conserved among species, which synergistically contribute to maintain physiological sodium wasting and weigh loss in every newborn.

Further investigations of MR regulatory mechanisms, particularly in the neonatal period, could improve our understanding of the physiological mineralocorticoid resistance observed at birth, and might open pharmacological options for the management of sodium wasting in preterms, the most concerned by this pathology.

Acknowledgments

The authors are indebted to Pr Celso Gomez-Sanchez (University of Mississipi, USA) for his generous gift of anti-MR antibodies. The assistance of Meriem Messina (Inserm U693) is also gratefully acknowledged. This work was supported by funds from Institut National de la Santé et de la Recherche Médicale and University Paris-Sud 11 (to ML) and from NIH DK081662 (to JML) and NIH DK20593 (Vanderbilt DRTC Hormone Core lab for aldosterone and corticosterone assays).

References

1. **Holtback U, Aperia AC** 2003 Molecular determinants of sodium and water balance during early human development. Semin Neonatol 8:291-299
2. **Herin P, Aperia A** 1994 Neonatal kidney, fluids, and electrolytes. Curr Opin Pediatr 6:154-157
3. **Modi N** 1988 Development of renal function. Br Med Bull 44:935-956
4. **Shaffer SG, Meade VM** 1989 Sodium balance and extracellular volume regulation in very low birth weight infants. J Pediatr 115:285-290
5. **Bassett MH, White PC, Rainey WE** 2004 The regulation of aldosterone synthase expression. Mol Cell Endocrinol 217:67-74
6. **Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M** 2007 The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. Nucl Recept Signal 5:e012
7. **Mick VE, Itani OA, Loftus RW, Husted RF, Schmidt TJ, Thomas CP** 2001 The alpha-subunit of the epithelial sodium channel is an aldosterone-induced transcript in mammalian collecting ducts, and this transcriptional response is mediated via distinct cis-elements in the 5'-flanking region of the gene. Mol Endocrinol 15:575-588
8. **Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C** 1988 Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet 2:986-989
9. **Funder JW, Pearce PT, Smith R, Smith AI** 1988 Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242:583-585
10. **Martinerie L, Pussard E, Foix-L'Helias L, Petit F, Cosson C, Boileau P, Lombes M** 2009 Physiological partial aldosterone resistance in human newborns. Pediatr Res 66:323-328
11. **Martinerie L, Viengchareun S, Delezoide AL, Jaubert F, Sinico M, Prevot S, Boileau P, Meduri G, Lombes M** 2009 Low renal mineralocorticoid receptor expression at birth contributes to partial aldosterone resistance in neonates. Endocrinology 150:4414-4424

12. **Lee G, Makhanova N, Caron K, Lopez ML, Gomez RA, Smithies O, Kim HS** 2005 Homeostatic responses in the adrenal cortex to the absence of aldosterone in mice. Endocrinology 146:2650-2656

13. **Makhanova N, Lee G, Takahashi N, Sequeira Lopez ML, Gomez RA, Kim HS, Smithies O** 2006 Kidney function in mice lacking aldosterone. Am J Physiol Renal Physiol 290:F61-69

14. **Luther JM, Wang Z, Ma J, Makhanova N, Kim HS, Brown NJ** 2009 Endogenous aldosterone contributes to acute angiotensin II-stimulated plasminogen activator inhibitor-1 and preproendothelin-1 expression in heart but not aorta. Endocrinology 150:2229-2236

15. **Gomez-Sanchez CE, de Rodriguez AF, Romero DG, Estess J, Warden MP, Gomez-Sanchez MT, Gomez-Sanchez EP** 2006 Development of a panel of monoclonal antibodies against the mineralocorticoid receptor. Endocrinology 147:1343-1348

16. **Bayard F, Ances IG, Tapper AJ, Weldon VV, Kowarski A, Migeon CJ** 1970 Transplacental passage and fetal secretion of aldosterone. J Clin Invest 49:1389-1393

17. **van Beek JP, Guan H, Julan L, Yang K** 2004 Glucocorticoids stimulate the expression of 11beta-hydroxysteroid dehydrogenase type 2 in cultured human placental trophoblast cells. J Clin Endocrinol Metab 89:5614-5621

18. **Verrey F** 2001 Sodium reabsorption in aldosterone-sensitive distal nephron: news and contributions from genetically engineered animals. Curr Opin Nephrol Hypertens 10:39-47

19. **Schulz-Baldes A, Berger S, Grahammer F, Warth R, Goldschmidt I, Peters J, Schutz G, Greger R, Bleich M** 2001 Induction of the epithelial Na+ channel via glucocorticoids in mineralocorticoid receptor knockout mice. Pflugers Arch 443:297-305

20. **Diederich S, Eigendorff E, Burkhardt P, Quinkler M, Bumke-Vogt C, Rochel M, Seidelmann D, Esperling P, Oelkers W, Bahr V** 2002 11beta-hydroxysteroid dehydrogenase types 1 and 2: an important pharmacokinetic determinant for the activity of synthetic mineralo- and glucocorticoids. J Clin Endocrinol Metab 87:5695-5701

21. **Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, Thomas CP** 2002 Glucocorticoid-stimulated lung epithelial Na(+) transport is associated with regulated ENaC and sgk1 expression. Am J Physiol Lung Cell Mol Physiol 282:L631-641

22. **Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ, Myers RM** 2009 Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. Genome Res 19:2163-2171

23. **Makhanova N, Sequeira-Lopez ML, Gomez RA, Kim HS, Smithies O** 2006 Disturbed homeostasis in sodium-restricted mice heterozygous and homozygous for aldosterone synthase gene disruption. Hypertension 48:1151-1159

24. **Berger S, Bleich M, Schmid W, Cole TJ, Peters J, Watanabe H, Kriz W, Warth R, Greger R, Schutz G** 1998 Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. Proc Natl Acad Sci U S A 95:9424-9429

25. **Bleich M, Warth R, Schmidt-Hieber M, Schulz-Baldes A, Hasselblatt P, Fisch D, Berger S, Kunzelmann K, Kriz W, Schutz G, Greger R** 1999 Rescue of the mineralocorticoid receptor knock-out mouse. Pflugers Arch 438:245-254

26. **Geller DS** 2005 Mineralocorticoid resistance. Clin Endocrinol (Oxf) 62:513-520

27. **Pascoe L, Curnow KM, Slutsker L, Rosler A, White PC** 1992 Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency. Proc Natl Acad Sci U S A 89:4996-5000

28. **White PC** 1994 Disorders of aldosterone biosynthesis and action. N Engl J Med 331:250-258

29. **White PC** 2009 Neonatal screening for congenital adrenal hyperplasia. Nat Rev Endocrinol 5:490-498

30. **Grossmann C, Gekle M** 2009 New aspects of rapid aldosterone signaling. Mol Cell Endocrinol 308:53-62

31. **Schmidt BM, Georgens AC, Martin N, Tillmann HC, Feuring M, Christ M, Wehling M** 2001 Interaction of rapid nongenomic cardiovascular aldosterone effects with the adrenergic system. J Clin Endocrinol Metab 86:761-767

32. **Funder JW** 2005 The nongenomic actions of aldosterone. Endocr Rev 26:313-321

33. **Zennaro MC, Le Menuet D, Lombes M** 1996 Characterization of the human mineralocorticoid receptor gene 5'-regulatory region: evidence for differential hormonal

regulation of two alternative promoters via nonclassical mechanisms. Mol Endocrinol 10:1549-1560

34. **Munier M, Meduri G, Viengchareun S, Leclerc P, Le Menuet D, Lombes M** 2010 Regulation of mineralocorticoid receptor expression during neuronal differentiation of murine embryonic stem cells. Endocrinology 151:2244-2254

Figure legends:

Figure 1: Aldosterone and corticosterone levels: Aldosterone and corticosterone levels were measured in $AS^{-/-}$ (KO) and WT mice (WT) at the day of birth (D0) and at postnatal day eight (D8). Results are expressed as the mean ± SEM of 6 different samples. *, $p < 0.05$.

Figure 2: Mineralocorticoid receptor expression: MR mRNA expression was measured in the kidneys of $AS^{-/-}$ (KO), $AS^{+/-}$ (HET) and wild type (WT) mice at the day of birth (D0) and at eight-postnatal days (D8). Relative mRNA levels were determined by qRT-PCR in at least 4 different samples of each genotype and developmental stage. Results, expressed as the ratio of attomoles of specific gene per fentomoles of 18S, are means ± SEM of three independent experiments and correspond to the relative expression compared with that obtained in $AS^{-/-}$ mice kidneys at D0, which was arbitrarily set at 1. *, $p < 0.05$; ***, $p < 0.001$.

Figure 3: 11βHSD2 and αENaC expression: 11βHSD2 and αENaC mRNA expression were measured in the kidney of $AS^{-/-}$ (KO), $AS^{+/-}$ (HET) and wild type (WT) mice on the day of birth (D0) and at eight-postnatal days (D8). Relative mRNA levels were determined by qRT-PCR in at least 4 different samples of each genotype and developmental stage. Results, expressed as the ratio of attomoles of specific gene per fentomole of 18S, are means ± SEM of three independent experiments and correspond to the relative expression compared with that obtained in $AS^{-/-}$ mice kidneys at D0, which was arbitrarily set at 1. NS : Not significant; ***, $p < 0.001$.

Figure 4: Organotypic cultures of kidneys from newborn (OCD0) or postnatal day eight (OCD8) wild type mice: A. Organotypic cultures were grown in epithelial medium for 7 days. After dissection, single glomeruli and nephrons can be individualized in the medium. On the first day of culture (1) epithelial-like tubular cells start growing. After seven days of culture (7) a layer of epithelial-like tubular cells is observed. These cells demonstrate specific features of epithelial cells and have the capacity to form domes (arrow). **B.** Immunodetection of MR protein in organotypic cultures,

after 7 days of culture in epithelial medium for OCD0, or after 7 days of culture in epithelial medium followed by 24 h in minimal medium (MM) and 24 h in either MM (Control), MM + Aldosterone 10^{-7} M (A-7) or MM + Aldosterone 10^{-7} M and Spironolactone 10^{-6} M (A-7+Spiro-6) treatment for OCD8. Immunoreactive MR is absent in OCD0, but still detectable in epithelial-like tubular cells of OCD8 after a week of culture. MR immunostaining is cytoplasmic in absence of aldosterone (Control) or in presence of the MR antagonist, spironolactone, (A-7+Spiro-6). MR expression in the epithelial cells is nuclear in presence of aldosterone (A-7). **C.** Relative MR mRNA expression was determined in OCD0 and OCD8 after 7 days of culture in epithelial medium, followed by 24 h in minimal medium (MM) and 24 h in either MM (Control), MM + Aldosterone 10^{-7} M (A-7) or MM + Dexamethasone 10^{-7} M (D-7) treatment. Results, expressed as the ratio of attomoles of specific gene per fentomole of 18S, are means ± SEM. The control condition was chosen as our statistical reference.