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► To cite this version:

Laetitia Martinerie, Say Viengchareun, Géri Meduri, Hyung-Suk Kim, James M. Luther, et al.. Aldosterone postnatally, but not at birth, is required for optimal induction of renal mineralocorticoid receptor expression and sodium reabsorption.: Aldosterone regulation of renal MR expression. *Endocrinology*, 2011, 152 (6), pp.2483-91. 10.1210/en.2010-1460 . inserm-00579433

HAL Id: inserm-00579433

<https://inserm.hal.science/inserm-00579433>

Submitted on 7 Apr 2011

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

3

4 **Abbreviated Title: Aldosterone regulation of renal MR expression**

5 Laetitia Martinerie^{1,2,6}, Say Viengchareun^{1,2}, Geri Meduri^{1,3}, Hyung-Suk Kim⁴, James M.
6 Luther⁵, Marc Lombès^{1,2,6,7}

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

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

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

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

13 ⁵ *Division of Clinical Pharmacology, Vanderbilt University Medical Center, Nashville, TN,*
14 *USA;*

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

17 ⁷ *PremUp Foundation, Paris, F-75006, France.*

18

19 *** Corresponding author:**

20

21 Dr Marc LOMBES, MD, PhD
22 Inserm U693, Faculté de Médecine Paris-Sud
23 63, rue Gabriel Péri, 94276 Le Kremlin Bicêtre Cedex France
24 Tel 33 1 49 59 67 09
25 Fax 33 1 49 59 67 32
26 Email: marc.lombes@u-psud.fr

27

28 Reprint request should be addressed to Dr Marc LOMBES

29

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

31

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

33

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

36

37 **Word count of abstract: 248**

38 **Word count: 3897**

39

40 **Abstract**

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

58

59 **Introduction**

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

66 Renal sodium reabsorption is mainly controlled by aldosterone, a steroid hormone synthesized in the
67 adrenal gland zona glomerulosa, secondary to renin stimulation *via* angiotensin II and to potassium
68 stimulation (5). In the distal nephron, aldosterone, by binding to its receptor, the mineralocorticoid
69 receptor (MR), a transcription factor (6), tightly regulates the expression and activity of several
70 transporting proteins implicated in sodium homeostasis, including the alpha subunit of the epithelial
71 sodium channel (α ENaC) (7). The selectivity of the mineralocorticoid signaling pathway in the
72 epithelial cells is controlled at a prereceptor level by the 11 β -hydroxysteroid dehydrogenase type 2
73 enzyme (11 β HSD2) which metabolizes cortisol (or corticosterone in rodents) into inactive
74 compounds, incapable of MR binding (8, 9). We have previously demonstrated that the neonatal
75 sodium wasting is related to a physiological transient renal aldosterone resistance (10). We have also
76 established that this physiological aldosterone resistance is associated in both mice and humans with a
77 low renal MR expression at birth, both at the mRNA and protein level (11). This low renal MR
78 expression in newborns is followed by a significant increase in the postnatal period, with a complete
79 renal tubular expression developed at 8 postnatal days in mice and during the first year of life in
80 humans, paralleling renal maturation. The underlying mechanisms, responsible for the low renal MR
81 expression at birth and its postnatal increase, are currently unknown. Since aldosterone levels are very
82 high at birth and have a tendency to decrease in the postnatal period, mirroring MR expression (11),
83 we hypothesized that these high hormonal levels could contribute to the low neonatal renal MR
84 expression. To investigate the role of aldosterone on neonatal MR regulation, we utilized the
85 aldosterone-synthase knock-out mouse model ($AS^{-/-}$). These transgenic mice were originally generated

86 by Hyung-Suk Kim, using standard gene targeting methods, with a final construct lacking exons 1, 2,
87 part of exon 3 and introns 1 and 2 of the AS gene (12), and have undetectable levels of plasma
88 aldosterone. AS^{-/-} genotype is compatible with fetal development but newborns fail to thrive
89 postnatally and about 30% die between day 7 and 28 (12). Adult AS^{-/-} mice are small, weigh 25% less
90 than wild type animals, have low blood pressure, abnormal electrolyte homeostasis (increased plasma
91 concentrations of K⁺, Ca²⁺ and Mg²⁺, decreased concentrations of HCO₃⁻ and Cl⁻ but no difference of
92 plasma Na⁺ level under normal diet) and altered water metabolism (higher urine output, decreased
93 urine osmolality, and impaired urine concentrating and diluting ability). Higher levels of plasma
94 corticosterone and strong activation of the renin-angiotensin system are observed. In contrast, AS^{+/-}
95 mice have normal plasma aldosterone concentrations, normal blood pressure and no electrolyte
96 disturbances (13). To evaluate whether renal MR expression in the neonatal period is dependent on
97 aldosterone, we have quantified and compared MR mRNA steady state levels in the kidneys of AS^{-/-},
98 AS^{+/-} and wild type littermates, using quantitative real-time PCR. To determine whether maternally-
99 derived aldosterone exposure *in utero* modifies MR expression at birth, we conducted two breeding
100 strategies, utilizing pups derived from either AS^{-/-} or AS^{+/-} mice. We have also analyzed the expression
101 of other actors of the mineralocorticoid (11βHSD2, αENaC) and glucocorticoid (GR) signaling
102 pathways, and measured hormonal status. Our results were subsequently confirmed *in vitro*, using
103 organotypic cultures of wild type newborn murine kidneys. We demonstrate that, whereas aldosterone
104 is dispensable for MR regulation at birth, it is pivotal for optimal induction of renal MR expression
105 and sodium homeostasis in the postnatal mature kidney, effects that cannot be compensated by the
106 glucocorticoid signaling pathway.

107

108 **Materials and Methods**

109

110 *Animals and Breeding strategy*

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

122

123 *Hormonal Analyses*

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

131

132 *Organotypic cultures*

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

136 Then, kidneys were manually dissected with a needle. Homogenates were pooled and centrifuged for 3
137 min at 300 g, supernatant was withdrawn and cellular pellet was resuspended in a specific epithelial
138 medium described below. Cellular suspensions were then seeded on collagen I-coated 12-well plates
139 (Collagen I from Institut Jacques Boy, France), and routinely cultured for seven days at 37°C in a
140 humidified incubator gassed with 5% CO₂ within an epithelial medium composed of DMEM/HAM's
141 F12 (1:1), 2 mM glutamine, 50 nM dexamethasone, 50 nM sodium selenite, 5 µg/ml transferrin, 5
142 µg/ml insulin, 10 ng/ml EGF, 2 nM T3, 100 U/ml penicillin/streptomycin, 20 mM HEPES, pH 7.4, 5%
143 dextran charcoal-treated serum and 1% amphotericin B (the latter added to the medium for the first
144 48 h of culture only).

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

150

151 *RT-PCR and Quantitative real-time PCR*

152 Total RNA was extracted from tissues or cells with the TRIZOL reagent (Invitrogen) according to the
153 manufacturer's recommendations, and RNA was thereafter processed for RT-PCR, as previously
154 described (11). One µg of total RNA, isolated from frozen samples, was subjected to DNase I
155 Amplification Grade treatment (Invitrogen) and then reverse-transcribed by use of High-Capacity
156 cDNA RT kit from Applied Biosystems (Courtaboeuf, France). Samples were diluted 10-fold then
157 1/20 of the reverse transcription reaction was used for qRT-PCR using the Fast SYBR[®] Green Master
158 Mix (ABI, Applied Biosystems) containing 300 nM of specific primers (supplemental Table). qPCR
159 was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA).
160 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.
161 For standards preparation, amplicons were purified from agarose gel and subcloned into pGEMT-easy
162 plasmid (Promega, Charbonnières-les-Bains, France) and sequenced to confirm the identity of each
163 sequence. Standard curves were generated using serial dilutions of linearized standard plasmids,

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

170

171 *Immunocytochemistry*

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

176

177 *Statistical analyses*

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

181

182

183 **Results**

184 *Breeding strategy*

185 We conducted a specific breeding strategy in order to obtain four different newborn mice with various
186 genotypes and distinct developmental endocrine patterns. We obtained AS^{-/-} pups that had never been
187 exposed to aldosterone, by mating female AS^{-/-} mice with AS^{-/-} male mice. We obtained AS^{-/-} pups
188 that had been exposed to maternal aldosterone, through transplacental crossing (16), by mating AS^{+/-}
189 female mice with AS^{+/-} male mice, which also generated AS^{+/-} littermates and wild type newborn mice.
190 No difference was observed between genotypes regarding weight at birth (1.23 ± 0.07 , 1.39 ± 0.02 ,
191 1.38 ± 0.04 , 1.42 ± 0.11 g, respectively), and at postnatal day eight (3.51 ± 0.23 , 3.54 ± 0.19 , $4.11 \pm$
192 0.17 , 4.02 ± 0.27 g, respectively). Kidneys were collected at birth (D0) and at postnatal-day eight
193 (D8). No renal structural abnormality was observed.

194

195 *Aldosterone and corticosterone levels*

196 Aldosterone and corticosterone levels were measured in AS^{-/-} and wild type (WT) newborn mice at the
197 time of kidney retrieval (Fig 1). As expected, aldosterone was undetectable in AS^{-/-} mice both at D0
198 and D8. In wild type mice, mean aldosterone level at birth was 466 ± 245 pg/ml and decreased at D8
199 (94 ± 38 pg/ml) although the difference did not reach significance ($p=0.18$). Interestingly, we found, in
200 AS^{-/-} animals both at birth and at D8, very high levels of corticosterone (374 ± 317 and 681 ± 376
201 ng/ml, respectively) were detected in comparison to wild type mice of the same developmental stage,
202 particularly at D8 ($p=0.04$). Analogous high levels of corticosterone have also been reported in AS^{-/-}
203 adult mice on normal sodium diet (13). One could hypothesize that these high corticosterone levels are
204 secreted to compensate for aldosterone deficiency.

205

206 *Mineralocorticoid receptor expression*

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

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

215

216 *11 β HSD2 and α ENaC expression*

217 We have previously demonstrated in WT mice (11), a parallel evolution throughout renal development
218 between MR, and both the 11 β HSD2 enzyme, which confers mineralocorticoid selectivity, and
219 α ENaC, a prototypal MR target gene. Since corticosterone levels were very high in $AS^{-/-}$ mice, we
220 wondered whether the expression profiles of 11 β HSD2 and α ENaC would be modified in the four
221 different genotypes. It has indeed been suggested that the glucocorticoid pathway could be implicated
222 in the regulation of these two genes (17-19). The question raised was whether corticosterone could
223 compensate for aldosterone deficiency in $AS^{-/-}$ mice by inducing α ENaC expression *via* binding to the
224 glucocorticoid receptor (GR). The latter is a transcription factor closely related to MR and also known
225 to activate gene expression implicated in sodium transport (18, 19). 11 β HSD2 and α ENaC mRNA
226 expression were therefore analyzed at D0 and D8 in all genotypes. 11 β HSD2 displayed an identical
227 expression profile to that of the MR, with a low expression at birth and a significant increase in the
228 postnatal period, independent of aldosterone status (Fig 3A). On the contrary, α ENaC mRNA
229 expression profile appeared strikingly different (Fig 3B). Indeed, α ENaC mRNA levels were very low
230 in $AS^{-/-}$ mice both at D0 and D8, with no significant postnatal increase. α ENaC expression evolved in
231 parallel with aldosterone levels, with a 1.5-fold and a 2-fold increase at birth in $AS^{+/+}$ and WT mice,
232 respectively, and a 2.5-fold and 3-fold induction at D8 in these same genotypes, compared to levels in
233 $AS^{-/-}$ mice at D0. Of interest, at D0, levels of α ENaC transcripts in $AS^{-/-}$ newborns, originating from
234 $AS^{+/+}$ x $AS^{+/+}$ mice, and thus prenatally exposed to aldosterone, were significantly higher than in non-
235 exposed $AS^{-/-}$ x $AS^{-/-}$ littermates, whereas α ENaC mRNA levels were identical at D8 in these animals.
236 These results strongly suggest that maternally biosynthesized aldosterone may have a significant
237 impact on fetal kidney α ENaC expression. Indeed, at D8, when circulating aldosterone is no longer

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

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

245

246 *Organotypic cultures*

247 We next decided to establish a reliable cell-based model to further investigate the hormonal regulatory
248 mechanisms of renal MR expression in the neonatal period. We thus chose to grow organotypic
249 cultures of WT newborn (OCD0) and postnatal-day eight (OCD8) mouse kidneys. As presented in Fig
250 4A, after manual kidney dissection, the nephronic tubules and glomeruli can be clearly visualized in
251 the epithelial medium. Thereafter, epithelial-like tubular cells start to proliferate from nephronic
252 fragments attached to the collagen I-coated plates. After seven days of culture, a full layer of renal
253 tubular cells is observed with the typical features of epithelial cells, most notably the capacity to form
254 domes. Moreover, as shown in Fig 4B, we were unable to immunodetect MR protein in OCD0 after 7
255 days of culture, while a specific immunolabeling was readily detected in the epithelial-like tubular
256 cells in OCD8, just like that observed in wild type kidneys (11). The cell immunolabelling was
257 intracytoplasmic in the absence of aldosterone (control), or in the presence of a MR antagonist,
258 spironolactone (A-7 + Spiro-6), whereas aldosterone alone (A-7) induced a clear nuclear translocation
259 of the activated MR. These results suggest that primary cultures of renal cells maintain the capacity to
260 express MR protein, and therefore constitute a valuable and reliable experimental cell-based model to
261 investigate the impact of aldosterone (or corticosteroid hormone) exposure on neonatal (D0 and D8)
262 control of renal MR expression.

263

264 *Impact of Aldosterone on gene expression in organotypic cultures*

265 To explore the impact of mineralocorticoid and glucocorticoid hormones on neonatal renal mRNA
266 expression, we incubated organotypic cultures of wild type D0 and D8 mouse kidneys with various
267 steroids (aldosterone or dexamethasone). No variation in MR mRNA levels was observed in OCD0
268 after 24 h treatment with either hormone (Fig 4C), providing additional support for the lack of
269 corticosteroid hormone effects on MR expression when comparing D0 AS^{-/-} and WT mice (see Fig 2).
270 However, our cell model was sensitive to glucocorticoid but not aldosterone action since
271 dexamethasone, a steroid compound that is not submitted to metabolic conversion by the 11 β HSD2
272 enzyme (20), was able to induce α ENaC expression (data not shown), most likely through
273 glucocorticoid receptor activation. Indeed, the absence of MR protein in OCD0 explains why
274 aldosterone is inefficient in inducing α ENaC expression. Finally, in sharp contrast, in OCD8 cultures,
275 clearly expressing MR protein, aldosterone, but not dexamethasone, was able to induce a significant 2-
276 fold increase of MR transcripts ($p < 0.01$) (Fig 4C). This result corroborates the *in vivo* findings
277 obtained in mouse models, revealing a significantly higher MR mRNA expression in WT than in AS^{-/-}
278 mice, particularly at D8.
279

280 Discussion

281 In the present paper, we demonstrate both *in vivo* and *in vitro*, that, at birth, aldosterone has no impact
282 on the renal expression of its specific MR receptor. Physiologically, renal MR expression is extremely
283 low during the neonatal period both in mice and humans, and increases drastically during the postnatal
284 period, in parallel with other actors of the mineralocorticoid pathway such as 11 β HSD2, which confers
285 mineralocorticoid selectivity, and α ENaC, a prototypal MR target gene required for apical sodium
286 entry and transepithelial reabsorption (11). Herein, we show that the lack of aldosterone biosynthesis
287 observed in AS^{-/-} mice does not modify MR expression profile at this specific period of development,
288 characterized by a persistent low expression at birth and a postnatal induction at D8. Similarly,
289 aldosterone withdrawal or administration does not induce MR mRNA and protein expression in
290 organotypic cultures of newborn kidneys collected on the day of birth.

291 Interestingly, unlike the MR and 11 β HSD2 mRNA expression profiles, which were independent of
292 aldosterone status, α ENaC mRNA levels positively correlated with the degree of aldosterone
293 exposure, following the sequence AS^{-/-} < AS^{+/-} < WT. Indeed, in the absence of aldosterone (in AS^{-/-}
294 newborns from AS^{-/-} mothers, and in AS^{-/-} eight-day-old mice), α ENaC transcripts levels were low at
295 birth and did not increase postnatally.

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

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

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

329 It seems particularly interesting that, all the components of the renal mineralocorticoid signaling
330 pathway (MR, $11\beta HSD2$ and $\alpha ENaC$) are physiologically downregulated during the first days of life,
331 both in mouse and human newborns. Therefore, these mechanisms concur to impair sodium
332 reabsorption and facilitate sodium wasting, this physiological condition being of particular importance
333 during these first days, regardless of aldosterone status. This dissociation between aldosterone and
334 mineralocorticoid signaling pathway activation could constitute a renal protection mechanism

335 preventing inappropriate sodium retention. Alternatively, high physiological aldosterone
336 concentrations could potentially be required for rapid non-genomic effects, as previously suggested
337 (30-32). Nevertheless, these observations could explain why deficiency of either aldosterone
338 biosynthesis or MR expression becomes critical after the first week of life only, both in mice and
339 humans.

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

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

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

363 **Acknowledgments**

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

369

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461 **Figure legends:**

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

466

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

474

475 **Figure 3: 11 β HSD2 and α ENaC expression:** 11 β HSD2 and α ENaC mRNA expression were
476 measured in the kidney of $AS^{-/-}$ (KO), $AS^{+/-}$ (HET) and wild type (WT) mice on the day of birth (D0)
477 and at eight-postnatal days (D8). Relative mRNA levels were determined by qRT-PCR in at least 4
478 different samples of each genotype and developmental stage. Results, expressed as the ratio of
479 attomoles of specific gene per femtomole of 18S, are means \pm SEM of three independent experiments
480 and correspond to the relative expression compared with that obtained in $AS^{-/-}$ mice kidneys at D0,
481 which was arbitrarily set at 1. NS : Not significant; ***, $p < 0.001$.

482

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

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

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