**Pomc Knockout Mice Have Secondary Hyperaldosteronism Despite an Absence of Adrenocorticotropic Hormone**

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Aldosterone production is controlled by angiotensin II, potassium, and ACTH. Mice lacking *Pomc* and its pituitary product ACTH have been reported to have absent or low aldosterone levels, suggesting that ACTH is required for normal aldosterone production. However, this is at odds with the clinical finding that human aldosterone deficiency is not a component of secondary adrenal insufficiency. To resolve this, we measured plasma and urine electrolytes, together with plasma aldosterone and renin activity, in *Pomc*\(^{-/-}\) mice. We found that these mice have secondary hyperaldosteronism (elevated aldosterone without suppression of renin activity), indicating that ACTH is not required for aldosterone production or release in vivo. Exogenous ACTH stimulates a further increase in aldosterone in *Pomc*\(^{-/-}\) mice, whereas angiotensin II has no effect, and the combination of angiotensin II and ACTH is no more potent than ACTH alone. These data suggest that aldosterone production and release in vivo do not require the action of ACTH during development or postnatal life and that secondary hyperaldosteronism in *Pomc*\(^{-/-}\) mice is a consequence of glucocorticoid deficiency. (*Endocrinology* 149: 681–686, 2008)

Three major types of hormones are produced by the adrenal cortex: glucocorticoids, mineralocorticoids, and (in humans) androgens. Glucocorticoids (cortisol in humans and corticosterone in rodents) are secreted from the zona fasciculata under the control of corticotropin (ACTH), whereas aldosterone is secreted from the zona glomerulosa under the principal control of the renin-angiotensin system, potassium (K\(^+\)), and to a lesser extent ACTH. Aldosterone’s main function is to maintain electrolyte and volume balance, which is achieved by its action on epithelial cells of the renal collecting duct and distal colon, where it promotes sodium (Na\(^+\)) absorption and K\(^+\) excretion.

Low blood pressure and low Na\(^+\) concentration in the macula densa of the renal distal tubule lead to renin secretion. The renin-angiotensin-aldosterone system is exquisitely sensitive to dietary sodium intake. Sodium restriction reduces renal and peripheral vascular responsiveness and enhances adrenal responsiveness to angiotensin II, whereas sodium excess has the opposite effect. Renin cleaves angiotensinogen to angiotensin I. Angiotensin I is cleaved by angiotensin converting enzyme to generate angiotensin II. The binding of angiotensin II to G\(_{i}\)-protein-coupled angiotensin I receptors (1–3) activates phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5 bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, leading to a release of Ca\(^{2+}\) from the endoplasmic reticulum. Angiotensin II-induced elevation of cytosolic Ca\(^{2+}\) ultimately causes increased aldosterone biosynthesis and secretion. ACTH, cleaved from its precursor proopiomelanocortin (*Pomc* in mice), stimulates cortisol and aldosterone production through binding to its adrenal-specific G\(_{s}\)-protein-coupled receptor, Mcr2, which interacts with adenylyl cyclase to form the second messenger cAMP, leading to activation of cAMP-dependent protein kinase A (4). Although angiotensin II and ACTH use different second messengers, it has been shown by several groups that their signaling pathways in the adrenal are interrelated (5–7).

In secondary adrenal insufficiency in humans, due to either hypothalamic or pituitary disease, the renin-angiotensin-aldosterone-axis remains intact, and mineralocorticoid deficiency is not seen. However, in primary adrenal insufficiency, glucocorticoid deficiency and mineralocorticoid deficiency usually coexist. Several genetic mouse models of either primary or secondary adrenal insufficiency exist. As examples of primary adrenal defects, mice that are deficient in the transcription factor steroidogenic factor 1 (Sf1) completely lack adrenals and die within several days after birth with hypovolemia and aldosterone deficiency (8). H-2(aw18) mice with a homozygous deletion of approximately 80 kb in the region of chromosome 17, which includes the *Cyp21a2* (21 hydroxylase) gene, die soon after birth of presumed glucocorticoid and aldosterone deficiency (9, 10). Mineralocorticoid receptor (*Nr3c2*) knockout mice, which specifically lack mineralocorticoid function, survive only if saline is injected until weaning (11, 12). In contrast, CRH-deficient animals, which have secondary adrenal insufficiency and very low basal corticosterone levels coexist with normal aldosterone levels and are viable without any therapy (13).

*Pomc*\(^{-/-}\) mice, which lack all Pomc-derived peptides, have secondary adrenal insufficiency, and survive without steroid or salt treatment. These mice have been described to have either absent (14) or very low (15, 16) aldosterone levels. This is at odds with the clinical teaching that aldosterone defi-
ciency is not a component of secondary adrenal insufficiency. To resolve this, we measured plasma aldosterone, plasma renin activity, serum and urine electrolytes, and the aldosterone response to angiotensin II and ACTH in Pomc\(^{-/-}\) mice. We found that these mice have secondary hyperaldosteronism that responds further to ACTH treatment but not to angiotensin II alone.

**Materials and Methods**

**Animal housing**

Wild-type (Pomc\(^{+/-}\)) and Pomc-deficient (Pomc\(^{-/-}\)) mice (a kind gift from U. Hochgeschwender) on a 129SvEv x C57BL/6 background were housed on a 12-h light, 12-h dark cycle (lights off at 0700 h) with ad libitum access to standard rodent chow (Prolab RMH 3000; Labdiet, Richmond, IN) with normal sodium content (0.26% sodium) (17) and water. Daily salt intake was 11 ± 0.82 mg/d in Pomc\(^{+/-}\) mice and 17 ± 0.85 mg/d in Pomc\(^{-/-}\) mice. Both genotypes were obtained from the same heterozygous breedings. All experiments were performed in mice 3 months of age. Animals were housed individually 3 d before each experiment. Animal housing and care was done according to National Institutes of Health guidelines, and all experiments were approved by the Animal Care and Use Committee of Children’s Hospital Boston.

**Genotyping by PCR**

The Pomc genotype of each mouse was identified by PCR amplification of tail genomic DNA using primers (Biosource International, Camarillo, CA) specific for the Pomc\(^{-/-}\) and Pomc\(^{-/-}\) alleles in a single reaction. Pomc\(^{+/-}\) primers were forward 5’-GCGTGGATGCCGTGGCAAAACT-3’ and reverse 5’-AGCAAGGTGGGTACACCTTC-3’; Pomc\(^{-/-}\) primers were forward 5’-ACCTCCCCCTGAACCTCGCAATA-3’, reverse 5’-TCTGAGTTGGCAGGGTAGCA-3’. PCR were performed in a 50-μl volume containing 1× PCR buffer, deoxyribonucleotides (Roche Molecular Biochemicals, Indianapolis, IN) at a final concentration of 0.2 mM each, 30 pmol of the specific primers, and Tag DNA polymerase (PGC Scientifics, Gaithersburg, MD). PCR conditions were initial denaturation at 94 C for 4 min followed by 35 cycles of 94 C for 1 min, 62 C for 1 min, 72 C for 1 min, followed by 72 C for 6 min. PCR products were analyzed by electrophoresis through 1.3% agarose gels. With this method, a 317-bp DNA fragment is generated from the Pomc\(^{-/-}\) allele (Fig. 1).

**Blood collection and analysis**

Blood sampling for plasma aldosterone measurements was rapidly (within 30 sec of touching an animal cage) performed without anesthesia by retroorbital sinus phlebotomy using heparinized capillary tubes. Basal levels were collected at 0800 h. To obtain plasma samples, whole blood was centrifuged at 3000 rpm at 4 C for 10 min. Plasma was stored at −80 C until use. Serum electrolytes were analyzed by the clinical chemistry laboratory of Children’s Hospital Boston using ion-selective electrodes.

**Urine collection**

Urine was collected over a 24-h period while mice were housed in metabolic cages. Mice were acclimated to the metabolic cages for 24 h before urine collection. Urine electrolytes were analyzed by the clinical chemistry laboratory of Children’s Hospital Boston using ion-selective electrodes.

**Hormone analysis**

Aldosterone was measured using a commercially available RIA (Adaltis, Casalecchio di Reno, Italy) according to the manufacturer’s instructions. The detection limit of the assay was 6.0 pg/ml, and cross-reactivity with corticosterone was 0.002%. At this level of cross-reactivity, a corticosterone level of 10 μg/dl would appear as 2 pg/ml in the aldosterone assay, far below the aldosterone levels detected in this study. Samples, 25 μl each, were run in duplicate.

Plasma corticosterone was measured using a commercially available RIA (MP Biomedical, Orangeburg, NY) according to the manufacturer’s instructions. The detection limit of the assay was 0.77 μg/dl.

Plasma renin activity was measured as the amount of angiotensin I generated after incubation with excess angiotensinogen and plasma (18, 19). Five microliters of plasma were incubated with excess porcine angiotensinogen (5 μmol/liter; Sigma, St. Louis, MO) in a 24-μl reaction containing 7 μl Tris-maleate buffer (0.2 mol/l, pH 6.0), 2 μl maleate generation buffer (Diasorin, Stillwater, MN), 0.2 μl phenylmethylsulfonyl fluoride (Diasorin). After removal and freezing of 10 μl to determine background angiotensin I levels for each plasma sample, the remaining volume was incubated for 1 h at 37 C. Angiotensin I generation rate (nanograms per milliliter per hour) was then measured using a RIA (Gammacoat; Diasorin).

**Hormone administration**

To stimulate adrenal aldosterone secretion, mice were injected ip with either 10 μg/kg body weight of synthetic ACTH (Cortrosyn; Amphastar Pharmaceuticals, Rancho Cucamonga, CA) alone (16, 20), 50 μg/kg body weight of human angiotensin II (Ciba, Basel, Switzerland) alone, or with a combination of the same concentrations of synthetic ACTH and angiotensin II. Blood was collected 30 min after hormone administration. Glucocorticoid treatment of Pomc\(^{-/-}\) and wild-type mice was carried out by injecting animals with 20 μg/kg body weight dexamethasone (Roche Laboratories, Columbus, OH), which equals approximately twice the physiological glucocorticoid replacement dose (21), once daily for 3 consecutive days. Blood for aldosterone and renin activity was collected on the fourth day at 0800 h.

**Statistics**

For comparison of more than two groups, data were analyzed by two-way ANOVA, followed by Bonferroni/Dunn post hoc multiple com-

![Fig. 1. Agarose gel electrophoresis of PCR products of tail genomic DNA derived from Pomc\(^{-/-}\) (−/− lane), Pomc\(^{+/-}\) (+/+ lane), and Pomc\(^{+/-}\) (+/− lane) animals. A 600-bp band represents the knockout (KO) allele, and a 317-bp band represents the wild-type (WT) allele. PCR without added DNA (H2O lane) yielded only a primer band (at bottom of gel), as judged by the DNA ladder fragments (marker lane).](https://academic.oup.com/endo/article-abstract/149/2/681/2454836/fig1)
parison test. For comparison of two groups, data were analyzed by two-sided Student’s t test. A P value less than 0.05 was considered statistically significant. All data are presented as mean ± sem. Because no difference was observed between the genders of each genotype (data not shown), data from both genders of a given genotype and treatment group were pooled. In figures, groups that are statistically different from each other are denoted by the same lowercase letter.

Results

Urine and serum electrolytes (Na\(^+\) and K\(^+\)) in Pomp\(^{-/-}\) mice

To determine the effect of ACTH deficiency upon aldosterone action, we measured urinary Na\(^+\)/K\(^+\) ratio, which is inversely proportional to aldosterone’s effect in the kidney (22) in Pomp\(^{-/-}\) and Pomp\(^{+/+}\) mice (Fig. 2). The Na\(^+\)/K\(^+\) ratio assayed over a 24-h period was not significantly different between the two genotypes (Fig. 2A). Interestingly, despite the lack of ACTH, the Na\(^+\)/K\(^+\) ratio was, if anything, lower in Pomp\(^{-/-}\) compared with Pomp\(^{+/+}\) mice, indicating normal or possibly increased aldosterone function in the ACTH-deficient animals. There was no difference in serum Na\(^+\) or K\(^+\) concentrations between the genotypes (Fig. 2, B and C). The hemoglobin content of all serum samples was less than 100 mg/dl, indicating that hemolysis did not occur, which would have artificially elevated the potassium levels.

Basal plasma aldosterone and renin activity levels were measured at 0800 3 d after the individual housing of mice. Pomp\(^{-/-}\) mice displayed significantly higher aldosterone levels compared with Pomp\(^{+/+}\) mice (Fig. 3A). Of the three potential causes for hyperaldosteronism in Pomp\(^{-/-}\) mice, we ruled out elevated ACTH and hyperkalemia, because these mice lack ACTH, and their plasma K\(^+\) was normal (Fig. 2C). However, plasma renin activity, rather than being suppressed as it would be with a primary elevation in aldosterone, showed a trend toward being elevated (Fig. 3B), although not significantly so, compared with Pomp\(^{+/+}\) mice, consistent with secondary hyperaldosteronism.

Because Pomp\(^{-/-}\) mice are glucocorticoid deficient, elevated renin activity and aldosterone levels might compensate for the loss of glucocorticoid-mediated regulation of
vascular tone. Therefore, we determined whether the increased basal aldosterone and renin activity concentrations in Pomc<sup>−/−</sup> mice persisted after glucocorticoid treatment with dexamethasone, 20 µg/kg body weight ip for 3 d. Dexamethasone was chosen because its glucocorticoid potency is approximately 20-fold greater than that of cortisol, whereas its mineralocorticoid potency is approximately 1% that of aldosterone (23). Body weights of Pomc<sup>−/−</sup> and Pomc<sup>+/+</sup> animals before treatment were 24 ± 0.5 and 35.55 ± 3.5 g, respectively, and did not change significantly after dexamethasone treatment. After dexamethasone treatment, aldosterone levels in Pomc<sup>−/−</sup> mice were indistinguishable from those of Pomc<sup>+/+</sup> mice (Fig. 3C). Renin activity fell by approximately 40% compared with untreated Pomc<sup>−/−</sup> mice but was significantly greater than that in dexamethasone-treated Pomc<sup>+/+</sup> mice (Fig. 3D). The results of this experiment suggest that secondary hyperaldosteronism in Pomc<sup>−/−</sup> mice is due, at least in part, to glucocorticoid deficiency.

Aldosterone response after ACTH and angiotensin II stimulation

Aldosterone is stimulated by both angiotensin II and ACTH, but the interdependency between these two stimuli is not well understood. We therefore tested the aldosterone response to ACTH, angiotensin II, or both hormones. As before (Fig. 3A), basal levels of aldosterone were higher in Pomc<sup>−/−</sup> compared with normal mice (Fig. 4). ACTH injection (10 µg/kg) increased aldosterone levels in both genotypes, albeit to a lesser extent in Pomc<sup>−/−</sup> mice. In Pomc<sup>+/+</sup> mice, corticosterone levels increased from 1.3 ± 0.2 µg/dl at baseline to 56.1 ± 9.1 µg/dl after ACTH administration. In Pomc<sup>−/−</sup> animals, basal as well as ACTH-stimulated corticosterone levels were below the detection limit of the assay. After angiotensin II administration, aldosterone levels rose significantly in Pomc<sup>+/+</sup> mice, whereas Pomc<sup>−/−</sup> animals showed no response. The lack of response of Pomc<sup>−/−</sup> mice to exogenous angiotensin II could be evidence of preexisting elevation of endogenous angiotensin II (consistent with elevated renin activity, Fig. 3B) or might indicate a requirement of ACTH for the acute aldosterone response to angiotensin II. To help distinguish between these two possibilities, mice were given combined stimulation with angiotensin II and ACTH. After this, aldosterone levels in Pomc<sup>−/−</sup> mice, although significantly higher compared with basal levels, did not rise much further than that seen with ACTH alone. This suggests that the absent response to exogenous angiotensin II alone in Pomc<sup>−/−</sup> mice was not due to a requirement of ACTH for an acute response to angiotensin II but more likely to a preexisting maximal stimulation by endogenous angiotensin II. Taken together, our data suggest that Pomc<sup>−/−</sup> mice have an intact renin-angiotensin-aldosterone system and have secondary hyperaldosteronism as a consequence of isolated glucocorticoid deficiency.

Discussion

In this study, we found that Pomc<sup>−/−</sup> mice in the basal state have secondary hyperaldosteronism, with elevated aldosterone levels and no suppression of plasma renin activity, despite an absence of ACTH. Aldosterone and renin activity levels decrease after dexamethasone treatment, suggesting that glucocorticoid deficiency contributes to their secondary hyperaldosteronism. Aldosterone secretion in Pomc<sup>−/−</sup> mice responds further to exogenous ACTH but not to angiotensin II, possibly because angiotensin II is already elevated in the setting of secondary hyperaldosteronism.

In contrast to mice lacking Sf1 or mineralocorticoid receptor, which are completely deficient in mineralocorticoid function, Pomc<sup>−/−</sup> mice survive without any salt or mineralocorticoid supplementation. Initially, Pomc<sup>−/−</sup> mice had been described as having no adrenals and completely lacking corticosterone and aldosterone (14). Subsequently, highly atrophic adrenals with a distinctive zona glomerulosa and zona fasciculata have been detected in newborn and young knockout animals (15, 16). Reduced aldosterone levels, approximately 25% of wild-type, have been reported by these same investigators (15, 16). As the aldosterone values in wild-type mice measured by these two groups (15, 16) were approximately 10-fold higher than we and others have reported in rodents, it is possible that their elevated values were stress related and that the Pomc<sup>−/−</sup> mice were not capable of responding to the stress due to a lack of ACTH. Although lower dietary sodium levels might conceivably explain the elevated aldosterone levels in Pomc<sup>+/+</sup> animals described by these other investigators, we and they used mouse diets with similar sodium content. Alternatively, the timing of blood sampling might explain this difference. Because mice are nocturnal animals, they are active and eat mostly during the night and sleep and fast during the day. Other investigators collected blood for aldosterone measurement between 1600 and 1700 h (16). Therefore, low sodium intake throughout the day might have resulted in elevated aldosterone levels in wild-type mice in this group compared with our study, in which blood was collected at 0800 h.

Interestingly, we found normal serum potassium and so-
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Diurn levels in Pomc−/− mice, which suggests the presence of normal aldosterone function. A slightly lower urine Na+/K+ ratio in Pomc−/− mice compared with wild-type animals also indicates sufficient mineralocorticoid activity in the mutant genotype. Based upon these data, we were not surprised to find that basal aldosterone levels were not low. In fact, they were elevated. Aldosterone secretion is stimulated by three factors: potassium, ACTH, and the renin-angiotensin system. Pomc−/− mice are not hyperkalemic and lack ACTH, eliminating these possibilities. However, plasma renin activity is not suppressed despite hyperaldosteronism in Pomc−/− mice, indicating that they have secondary hyperaldosteronism.

Because Pomc−/− mice have higher body weights and food intake than Pomc+/+ animals (14), their daily salt intake on mouse chow was increased compared with Pomc+/+ animals (see Materials and Methods). Therefore, their secondary hyperaldosteronism cannot be explained by low dietary sodium intake. On the other hand, secondary adrenal insufficiency is associated with secondary hyperaldosteronism in humans (24). In that study, patients with secondary adrenal insufficiency had higher aldosterone and renin activity levels than normal persons on identical sodium intakes. Glucocorticoids participate in the maintenance of blood pressure and volume homeostasis, independent of their mineralocorticoid actions. It has been shown in vitro and ex vivo that glucocorticoids down-regulate endothelial nitric oxide (NO) III synthesis expression, resulting in a reduction of endothelial-derived NO, a potent vasodilator (25). Glucocorticoid deficiency would therefore lead to an increase in endothelial-derived NO and consequently to a decrease in systemic blood pressure. Low blood pressure could then activate the renin-angiotensin-system leading to elevated aldosterone secretion. Elevated plasma renin activity in Pomc−/− mice, which normalizes after treatment with dexamethasone, supports this hypothesis.

Dexamethasone is known to increase kaliuresis in adrenalectomized rats (26). This action of dexamethasone is not likely to be a mineralocorticoid effect, both because of its poor ability to activate the mineralocorticoid receptor and because it appears to act via a vascular rather than a direct tubular effect (27). This may in part explain why, despite a 3-fold elevation in basal plasma aldosterone, glucocorticoid-deficient Pomc−/− mice have only a trend toward increased kaliuresis (reflected by the urinary Na/K ratio, Fig. 2A) and normal plasma potassium (Fig. 2B). Moreover, the fact that in patients with Addison’s disease dexamethasone improves potassium excretion without concomitant sodium retention supports an effect not involving the mineralocorticoid receptor (28). Our observation that treatment of Pomc−/− mice with dexamethasone resulted in a decrease of aldosterone levels to normal supports the hypothesis that elevated basal aldosterone levels are likely related to the glucocorticoid deficiency in Pomc−/− mice.

Pomc+/+ mice display a robust aldosterone response to either ACTH or angiotensin II alone. In contrast, we found that in Pomc−/− mice, ACTH, but not angiotensin II, further stimulated the elevated basal aldosterone levels and that the combined administration of angiotensin II and ACTH was no more effective than ACTH alone. An elevation in endoge-
References


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