TASK channel deletion in mice causes primary hyperaldosteronism

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When inappropriate for salt status, the mineralocorticoid aldosterone induces cardiac and renal injury. Autonomous overproduction of aldosterone from the adrenal zona glomerulosa (ZG) is also the most frequent cause of secondary hypertension. Yet, the etiology of nontumorigenic primary hyperaldosteronism caused by bilateral idiopathic hyperaldosteronism remains unknown. Here, we show that genetic deletion of TWIK-related acid-sensitive K (TASK)-1 and TASK-3 channels removes an important background K current that results in a marked depolarization of ZG cell membrane potential. Although TASK channel deletion mice (TASK^{-/-}) adjust urinary Na excretion and aldosterone production to match Na intake, they produce more aldosterone than control mice across the range of Na intake. Overproduction of aldosterone is not the result of enhanced activity of the renin-angiotensin system because circulating renin concentrations remain either unchanged or lower than those of control mice at each level of Na intake. In addition, TASK^{-/-} mice fail to suppress aldosterone production in response to dietary Na loading. Autonomous aldosterone production is also demonstrated by the failure of an angiotensin type 1 receptor blocker, candesartan, to normalize aldosterone production to control levels in TASK^{-/-} mice. Thus, TASK^{-/-} channel knockout mice exhibit the hallmarks of primary hyperaldosteronism. Our studies establish an animal model of nontumorigenic primary hyperaldosteronism and identify TASK channels as a possible therapeutic target for primary hyperaldosteronism.

potassium channels | adrenal glomerulosa cells | renin | aldosterone

Primary hyperaldosteronism (PA), the most common cause of secondary hypertension, is a direct consequence of autonomous aldosterone overproduction. In patients with PA, plasma aldosterone is inappropriately high for circulating levels of plasma renin and is not normalized by dietary or drug treatments that suppress the renin-angiotensin system (RAS) (1–5). Thus, in PA, neither volume expansion induced by dietary sodium loading nor the use of antihypertensive agents that either antagonize the angiotensin type 1 (AT₁) receptor (angiotensin receptor blockers) or block the generation of angiotensin II (Ang II) (angiotensin-converting enzyme inhibitors) remedies aldosterone excess (6).

Strikingly, among patients that are resistant to combination antihypertensive therapy, the prevalence of PA is estimated to be ≈20% worldwide (3, 6, 7). The incidence of PA increases with the severity of hypertension, and among resistant hypertensives, mineralocorticoid receptor blockade is of broad benefit (8–10). Among the seven diagnosed subtypes of PA, aldosterone-producing adenoma (APA) and bilateral idiopathic primary hyperaldosteronism (IHA) are of equivalent prevalence, together accounting for 95% of diagnosed cases (5). Unlike APA of known etiology that is correctable by surgery, the pathophysiology underlying nontumorigenic IHA is unknown, and specific therapies to reduce aldosterone production are not clinically available (5).

Circulating aldosterone is produced from the adrenal gland by cells of the zona glomerulosa (ZG). Ang II and extracellular K are potent independent regulators of aldosterone production,

yet the strength of these agonists in the physiological setting depends on their combined activities and their synergy (11–14). Small elevations in plasma potassium within the physiological range increase the sensitivity of ZG cells to stimulation by Ang II (13, 15). In this setting, both K and Ang II mediate membrane depolarization of ZG cells (14, 16) to enhance the opening of Ang II-modulated, low-voltage-activated, Ca channels (Cav3.2) whose activity underlies the *in vivo* control of aldosterone production (17, 18).

Adrenal ZG cells maintain a hyperpolarized resting membrane voltage that is negative to –70 mV (14, 16, 19). Although macroscopic K currents recorded from rat, bovine, and human ZG cells have revealed the expression of multiple voltage-dependent K conductances, the mRNA for TWIK-related acid-sensitive K (TASK)-1 and TASK-3 subunits is particularly abundant (20–23). TASK-1 (KCNK-3, K2P3.1) and TASK-3 (KCNK-9, K2P9.1) are two closely related family members of the KCNK family of two-pore domain/four-transmembrane (2P/4TMS) K channels that form background or “leak” K channels. These channels show little or no voltage dependence and only mild rectification properties in physiological asymmetric K solutions. By generating leak or background K currents, they play a principal role in setting negative membrane voltages (24, 25). TASK-1 and TASK-3 channels are inhibited by extracellular protons in the physiological range and by hormones that activate G_q-coupled receptors (26). Direct inhibition of TASK-1 or TASK-3 channels by activation of the AT_{1A} or AT_{1B} receptor or deletion of these channels from ZG cells would be expected to depolarize ZG cell membrane potential and produce an increase in voltage-dependent Ca channel activity that drives the production of aldosterone (14).

In this work, we used TASK subunit knockout mice and developed a method for recording from ZG cells in mouse adrenal slices to identify a TASK channel contribution to background K currents and membrane potential in ZG cells. In addition, we examined the role of TASK channels in the control of aldosterone production *in vivo*. Our results establish that genetic deletion of TASK-1 and TASK-3 channels from mice causes ZG membrane depolarization and elicits autonomous overproduction of aldosterone. These TASK knockout mice represent an animal model of PA that recapitulates key characteristics of IHA.

Results

Targeting and Successful Knockout of TASK-1 and TASK-3 Genes. To examine contributions of TASK channels to background K

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Fig. 1. Generation and initial characterization of a TASK^{-/-} mouse line. (A) Targeting constructs containing a neo cassette and loxP sites flanking the second exon of each TASK channel gene were used to generate mice bearing floxed TASK alleles that were crossed with a deleter-cre strain. Progeny were intercrossed to generate wild-type (+/+), heterozygous (+/-), or homozygous (-/-) knockout mice. (B) Successful excision of exon 2, validated by Southern blotting of tail DNA (digested with BamHI) and multiplex PCR analysis using the indicated primers, removes all channel coding regions downstream of the selectivity filter in the first pore domain. (C) Detection of TASK-1 (Upper) and TASK-3 (Lower) mRNA in adrenal slices from control (Left) and TASK^{-/-} mice (Right) by *in situ* hybridization with specific [³³P]cRNA probes.

currents in adrenal ZG cells and to test their role in the control of aldosterone production, we created mice in which both TASK-1 and TASK-3 were deleted (TASK-1^{-/-}:TASK-3^{-/-}; hereafter called TASK^{-/-}). As described in ref. 27 (see also *Methods*), the second exon of each TASK channel gene was “floxed” (Fig. 1A), and knockin animals bearing floxed alleles were generated by standard procedures. These mice were crossed with a deleter-cre mouse line to excise the floxed alleles; successful recombination and germ-line transmission confirmed by Southern blotting (Fig. 1B, Upper) and by multiplex PCR (Fig. 1B, Lower). As reported, TASK^{-/-} knockout mice were viable and showed no obvious sensorimotor deficits in several standard behavioral tests (e.g., rotarod, wire hang, tail flick) (27). For these studies, we used adult male mice (≈4 months old).

To examine the distribution and abundance of mRNA for TASK-1 and TASK-3 subunits in adrenal glands of control and knockout mice, we performed *in situ* hybridization on mouse adrenal slices with ³³P-labeled cRNA probes complementary to TASK-1 and TASK-3 mRNA (Fig. 1C). In adrenal glands from control mice (Fig. 1C, Left), TASK-1 mRNA was found throughout the cortex in the ZG and zona fasciculata, with little to no expression in the zona medullaris (Fig. 1C, Upper Left). By contrast, TASK-3 transcripts were localized primarily to the outer ZG and the inner zona medullaris and were absent from the zona fasciculata (25) (Fig. 1C, Lower Left); as expected, neither TASK-1 nor TASK-3 mRNA was expressed in adrenal glands of TASK^{-/-} mice (Fig. 1C, Right).

Cellular morphology and zonation were preserved within adrenal glands of adult male TASK^{-/-} mice (Fig. 2A, Right). Cell clusters characteristic of ZG cells were evident immediately beneath the capsular envelope in toluidine blue-stained adrenal

Fig. 2. Adrenal glands from TASK^{-/-} mice have normal cellular organization and zonation. Tissue slices were prepared from paraformaldehyde-fixed adrenal glands excised from control (Left) and TASK^{-/-} (Right) mice. (A Upper) Toluidine blue staining reveals dense ZG cell clusters beneath the capsular envelope (Magnification: ×40.) (Lower) Cellular organization differs among the cells of the zona fasciculata (ZF) and the zona medullaris (ZM). (Magnification: ×10.) (B) Distribution of mRNA transcripts of Cyp11b1 (11β-hydroxylase, Upper) and Cyp11b2 (aldosterone synthase, Lower) in adrenal slices from control (Left) and TASK^{-/-} mice (Right) by *in situ* hybridization with specific digoxigenin-labeled cRNA probes. [Scale bars: 50 μm (A Upper); 200 μm (A Lower and B).]

slices from both control (Fig. 2A, Upper Left) and TASK^{-/-} (Fig. 2A, Upper Right) mice; likewise, in both control and TASK^{-/-} mice, cells radiate centripetally within the zona fasciculata and cluster nonuniformly within the zona medullaris (Fig. 2A, Lower Left and Right). As a further test of appropriate adrenal structure in TASK^{-/-} mice, we evaluated adrenal expression of Cyp11b1 (11β-hydroxylase; Fig. 2B, Upper) and Cyp11b2 (aldosterone synthase; Fig. 2B, Lower) by using nonisotopic *in situ* hybridization. In both control (Left) and TASK^{-/-} knockout (Right) mice, Cyp11b1 transcripts were preferentially expressed in the zona fasciculata and reticularis, in contrast to Cyp11b2 mRNA that was localized primarily in the ZG. These data indicate that zone-specific expression of CYP11b1 and CYP11b2 was unaltered in TASK^{-/-} mice; they provide added indication that there are no overt abnormalities in adrenal gland structure that arise from TASK channel deletion, and they support the use of our conventional TASK knockout lines in the proposed studies. Interestingly, a recent study reports sex differences in effects of TASK-1 subunit deletion on adrenal development and zonation; males appear normal, and females show marked developmental abnormalities (25). Our data extend these observations showing that adrenals from male mice lacking both TASK-1 and TASK-3 are also normal in morphology and zonation.

TASK Channel Deletion Depolarizes Adrenal Glomerulosa Cells. To date there have been no direct measurements of TASK channel currents in mouse adrenal ZG cells *in situ*. We developed an adrenal slice preparation from adult mice (>P60) to obtain whole-cell recordings from these cells. This slice approach allows unambiguous identification of ZG cells based on their location in subcapsular cell rosettes (Fig. 3A) and obviates concerns with yield and identification of the small fraction of ZG cells anticipated with collagenase-assisted dissociation approaches. As illustrated for the Lucifer yellow-filled cell in Fig. 3A, we recorded from directly visualized ZG cells in concentric glomeruli just beneath the capsular layer (Fig. 3A, demarcated by arrows) and found that ZG cells from TASK^{-/-} mice display altered electrophysiological properties, consistent with TASK channel contributions to background K currents in these cells. In control tissue, ZG cell currents showed pharmacological and voltage-dependent properties characteristic of pH- and anesthetic-sensitive TASK channels (24, 28–30), i.e., a substantial outward current at the holding potential ($V_h = -60$ mV; Fig. 3

Fig. 3. TASK channel currents control ZG cell membrane potential. (A) Adrenal slice visualized under Nomarski optics showing a cluster of glomerulosa cells (delineated by arrows) and adjacent Lucifer yellow-filled patch pipette sealed onto a recorded cell. (B and C) Whole-cell current at $V_h = -60$ mV and during ramp depolarizations in ZG cell from control (Upper) and TASK^{-/-} mice (Lower). A weakly rectifying pH- and halothane-sensitive current was absent from TASK^{-/-} ZG cells. (D) Outward holding current and effects of bath pH on current in ZG cells from control animals were absent in TASK^{-/-} mice (interaction main effect: $F_{2,8} = 6.07$, $P < 0.025$, by two-way RM-ANOVA). (E) Membrane potential was more depolarized (by ≈ 20 mV) in ZG cells from TASK^{-/-} mice ($n = 5$ per genotype; *, $P < 0.001$).

B and D) that was reduced on bath acidification but enhanced on bath alkalization and/or exposure to halothane (Fig. 3B–D). The pH-sensitive current (Fig. 3C) and the halothane-sensitive current (data not shown) reversed near E_K and displayed a weakly rectifying I - V profile. In TASK^{-/-} mice, these pH- and anesthetic-sensitive TASK-like background currents were conspicuously absent (Fig. 3B–D). Thus, TASK channels conduct a background K current in ZG cells. This current contributes to the negative membrane potential of ZG cells because membrane potential was significantly more depolarized in TASK^{-/-} mice than in control mice (control: -73.2 ± 2.5 mV, $n = 6$; TASK^{-/-}: -53.9 ± 1.0 mV, $n = 6$, $P < 0.05$; Fig. 3E).

TASK^{-/-} Mice Display Primary Hyperaldosteronism That Is Resistant to Salt Suppression. To assess whether TASK channel deletion alters aldosterone production *in vivo*, we studied age-matched control and TASK^{-/-} mice maintained (≥ 1 week) on diets with different Na content: normal salt (NS), 0.32%; low salt (LS), 0.05%; or high salt (HS), 4%. We evaluated urinary aldosterone excretion (24 h, normalized to creatinine) as a measure of integrated aldosterone secretory activity *in vivo* to minimize stress-induced aldosterone production and avoid the variability associated with diurnal patterns of aldosterone production (31).

TASK^{-/-} mice exhibit PA. Independent of salt ingestion,

TASK^{-/-} mice produced more aldosterone than control mice despite lower concentrations of plasma renin (PRC) (Fig. 4A). Consistent with stimulation of the RAS, dietary Na restriction (LS diet) elevated urinary aldosterone excretion above that produced on a NS diet in mice of both genotypes (control: 19 ± 3 to 90 ± 12 ng/mg, $n = 10$; TASK^{-/-}: 54 ± 17 to 165 ± 34 ng/mg, $n = 10$; Fig. 4A). Dysregulation of aldosterone production was most apparent in TASK^{-/-} mice during HS challenge when activity of the RAS is reduced. Indeed, by comparison with aldosterone production on a NS diet, control animals on a HS diet invariably decreased aldosterone output (19 ± 3 to 10 ± 1 ng/mg), whereas most TASK^{-/-} mice actually increased output (54 ± 17 to 79 ± 21 ng/mg; Fig. 4A and B). Moreover, TASK^{-/-} mice failed to suppress aldosterone production on a HS diet for at least 7 days, indicating a chronic failure of TASK^{-/-} to suppress production (Fig. 4C). Thus, TASK^{-/-} mice exhibit two hallmarks of PA: elevated levels of aldosterone despite low circulating renin, and failure to suppress aldosterone production by dietary salt ingestion (Fig. 4A and B).

AT₁ Receptor Blockade Does Not Normalize Aldosterone Production and Identifies Enhanced Sensitivity to Ang II. Excessive aldosterone production during Ang II receptor blockade represents a further observation in PA, which was also recapitulated in TASK^{-/-} mice. Mice were maintained on a LS diet to elevate aldosterone production, and an oral dose of the insurmountable AT₁R receptor blocker candesartan was administered in the drinking water [10 mg/kg/day; candesartan cilexetil (AstraZeneca)]. Candesartan suppressed aldosterone production in mice of both genotypes by $>75\%$, reaching a steady-state level of steroid excretion within 2 days of administration (Fig. 4D). However, despite the administration of a maximal oral dose of candesartan, aldosterone production from TASK^{-/-} mice remained elevated above that of control mice (control: 26 ± 1 ng/mg, $n = 3$; TASK^{-/-}: 69 ± 23 ng/mg, $n = 3$). Enhanced autonomous aldosterone production in TASK^{-/-} mice is highlighted further by normalizing mean steroidogenic responses (2–3 days) of control and TASK^{-/-} mice to that of control mice on a NS diet (Fig. 4E). AT₁ receptor blockade failed to normalize aldosterone production in TASK^{-/-} mice despite a lower plasma concentration of renin. In addition, these normalized data underscore a marked increase in the candesartan-sensitive component of aldosterone production in TASK^{-/-} mice (control: 5.0 ± 0.6 -fold response, $n = 3$; TASK^{-/-}: 20.0 ± 0.7 -fold response, $n = 3$, $P \leq 0.001$), indicating an enhanced sensitivity to Ang II in knockout animals. Nonetheless, despite aldosterone excess, TASK^{-/-} mice were able to adapt to achieve Na and K balance (Fig. 4F), as is observed in patients with PA (7). Like patients with PA, TASK^{-/-} mice were mildly hypokalemic across all diets ($P \approx 0.006$), with plasma values of K on the NS diet being reduced by $\approx 25\%$ (control: 4.2 ± 0.1 mM; TASK^{-/-}: 3.4 ± 0.2 mM, $n = 6$ per genotype, $P < 0.05$). This relative hypokalemia would contribute to the attainment of K balance in the steady state by reducing K secretion at the cortical collecting tubule.

Discussion

We find that genetic deletion of TASK-1 and TASK-3 subunits yields a mouse model of PA, and our data provide a cellular mechanism for excess aldosterone production in these animals. Previous work has established that aldosterone production from cells of the ZG is calcium-dependent, and elevation in intracellular free calcium is critical for stimulated production (32–34). Across species, low-voltage-activated Cav3.2 channels of the Cav3.0 family are the major calcium conductance of the ZG cell, and although mostly closed at rest, they are available for opening during cell depolarization (18). However, ZG cells do not support Na-dependent action potentials and maintain a very negative resting membrane voltage (14, 16, 19), at which the

Fig. 4. TASK^{-/-} mice produce excess aldosterone and exhibit characteristics of idiopathic primary hyperaldosteronism. Twenty-four-hour urine chemistries after 1 week on sodium diets were as follows: low Na (LS, 0.05%), normal Na (NS, 0.32%), high Na (HS, 4%). Mice were studied ($n = 10$ /genotype) in metabolic cages (7 days per diet): control (open circles) and TASK^{-/-} (filled circles). (A) Urinary aldosterone (Ualdosterone) normalized to creatinine ($\dagger$, genotype main effect, $F_{1,18} = 10.03$, $P < 0.005$) and plasma renin ($\dagger$, $F_{1,11} = 11.2$, $P < 0.005$). (B) Urinary aldosterone for each animal on HS, normalized to NS. Circles, mean values $\pm$ SEM on days 4–7 of HS; squares, mean of each genotype ($n = 10$). (C) Time course of urinary aldosterone on HS after LS ($\dagger$, $F_{1,18} = 12.45$, $P < 0.002$). (D) Time course of urinary aldosterone after candesartan administration to mice on LS ($\dagger$, $F_{1,4} = 8.53$, $P < 0.05$). (E) Steady-state urinary aldosterone ($\dagger$, $F_{1,4} = 39.15$, $P < 0.005$) and plasma renin ($\dagger$, $F_{1,4} = 39.1$, $P < 0.005$) expressed relative to control animals on NS diet; genotype differences are independent of treatment. (F) Urinary Na and K are not different between genotypes on any diet. ($\dagger$, F value for genotype main effect by two-way RM-ANOVA; *, $P < 0.05$, control vs. TASK^{-/-} on indicated treatment, Student–Neuman–Keuls post hoc test).

open probability of even Cav3.2 channels is very low (35). Our experiments show that TASK channels are a dominant K conductance of the ZG cell that serves to clamp the membrane to these hyperpolarized voltages, effectively restraining the production of aldosterone. Accordingly, the removal of TASK-1 and TASK-3 subunits from the adrenal ZG cell elicits an approximate 20-mV depolarization that brings the membrane voltage into a range where T-type channels are activated to increase intracellular calcium (35, 36). Under physiological conditions, this TASK channel-mediated restraint on aldosterone production is removed by activation of AT₁ receptors that release G_q to inhibit TASK channels (26).

Our experiments also show that the genetic deletion of these two critical background K channels results in autonomous aldosterone overproduction from ZG cells. Aldosterone production in TASK^{-/-} mice was inappropriate for the level of circulating renin, was not suppressed by oral sodium loading, and was not fully normalized by AT₁ receptor blockade, similar to responses of patients with PA. We also observed that aldosterone levels in TASK^{-/-} mice were extremely sensitive to changes in circulating levels of Ang II. Despite comparatively low levels of circulating renin in TASK^{-/-} mice, candesartan-inhibitable aldosterone production was 230% of that of control mice when RAS activity was increased on a low-salt diet (*cf.* LS and LS+C in Fig. 4E); differences in aldosterone production were less pronounced on the NS diet when RAS activity was lower (see NS in Fig. 4E). Interestingly, among patients with PA, enhanced Ang II-sensitive aldosterone production is characteristic of those with

IHA and not of those with true Conn's syndrome caused by APA (37). These differences in Ang II sensitivity are not apparent in comparisons between IHA and APA patients on an unrestricted salt diet (*i.e.*, when RAS activity is relatively suppressed), in whom the angiotensin-converting enzyme inhibitor captopril was equally effective at reducing plasma aldosterone levels (38). However, an exquisite differential sensitivity in aldosterone secretory responses to changes in circulating Ang II and [des-aspartyl¹]-Ang II (Ang III) is observed after peptide infusion or postural changes in patients with IHA but not APA (39–41). Taken together, the findings in this work provide proof of principle that an intrinsic alteration in the membrane properties of ZG cells can induce autonomous aldosterone production with clinical features typical of IHA, including increase responsiveness to Ang II (42).

Given that the incidence of diagnosed PA increases with the severity of hypertension (6), it was anticipated that TASK^{-/-} mice chronically overproducing aldosterone would also be hypertensive. This appears to be so in TASK^{-/-} mice (control: 99 ± 2 mmHg, $n = 6$; TASK^{-/-}: 120 ± 4 mmHg, $n = 3$, $P < 0.006$; N.A.G., unpublished telemetry data). Because our studies were performed in conventional TASK^{-/-} knockout mice, a contribution from other mechanisms of blood pressure control (43, 44) to the documented hypertension cannot be excluded. In fact, many patients with IHA require a second antihypertensive agent in addition to a mineralocorticoid receptor blocker to normalize blood pressure, implying that aldosterone excess may not be the sole causative factor (5). Nonetheless, the hypertension in

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