Urine concentrating defect in prostaglandin EP1-deficient mice

Chris R. J. Kennedy,1,2 Huaiqin Xiong,1 Sherine Rahal,1,2 Jacqueline Vanderluit,1 Ruth S. Slack,1 Yahua Zhang,3 Youfei Guan,3 Matthew D. Breyer,3 and Richard L. Hébert2

1Kidney Research Centre, Division of Nephrology, Department of Medicine, Ottawa Hospital, Ottawa Health Research Institute, 2Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada; and 3Division of Nephrology, Vanderbilt University Medical Center, Nashville, Tennessee

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Kennedy CR, Xiong H, Rahal S, Vanderluit J, Slack RS, Zhang Y, Guan Y, Breyer MD, Hébert RL. Urine concentrating defect in prostaglandin EP1-deficient mice. Am J Physiol Renal Physiol 292:F868–F875, 2007. First published August 1, 2006; doi:10.1152/ajprenal.00183.2005.—We investigated the role of the prostaglandin E2 (PGE2) EP1 receptor in modulating urine concentration as it is expressed along the renal collecting duct where arginine-vasopressin (AVP) exerts its anti-diuretic activity, and in the paraventricular and supraoptic nuclei of the hypothalamus where AVP is synthesized. The urine osmolality of EP1-null mice (EP1−/−) failed to match levels achieved by wild-type (WT) counterparts upon water deprivation (WD) for 24 h. This difference was reflected by higher plasma osmolality in WD EP1−/− mice. Along the collecting duct, the induction and subapical to plasma membrane translocation of the aquaporin-2 water channel in WD EP1−/− mice appeared equivalent to that of WD WT mice as determined by quantitative RT-PCR and immunohistochemistry. However, medullary interstitial osmolalities dropped significantly in EP1−/− mice following WD. Furthermore, urinary AVP levels of WD EP1−/− mice were significantly lower than those of WD WT mice. This deficit could be traced back to a blunted induction of hypothalamic AVP mRNA expression in WD EP1−/− mice as determined by quantitative RT-PCR and administration of AVP mimetic [deamino-Cys1, D-Arg8]−vasopressin restored a significant proportion of the urine concentrating ability of WD EP1−/− mice. When mice were water loaded to suppress endogenous AVP production, urine osmolalities increased equally for WT and EP1−/− mice. These data suggest that PGE2 modulates urine concentration by acting at EP1 receptors, not in the collecting duct, but within the hypothalamus to promote AVP synthesis in response to acute WD.

URINE CONCENTRATION IS A HIGHLY regulated process requiring the coordinated actions of a number of hormones at several tissue locales (2). The body responds to acute volume depletion, infection, or hyperosmolarity by stimulating the production of antidiuretic hormone, arginine-vasopressin (AVP), within the hypothalamic paraventricular (PVN) and supraoptic (SON) neurons. AVP is then processed, stored, and secreted into the venous flow by the posterior pituitary to interact with renal V2 receptors located in the principal cells of the collecting duct to promote aquaporin-2 (AQP2)-dependent water reabsorption (25). Many of these steps are subject to modulation by endocrine factors such as ANG II, catecholamines, as well as certain metabolites of the cyclooxygenase (COX) pathway, the prostaglandins (PGs) (2). Accordingly, renal prostaglandin E2 (PGE2) antagonizes the actions of AVP since NSAIDs transiently enhance urine concentration (1). Direct evidence for the underlying mechanism was provided by Hébert et al. (13) who demonstrated that PGE2 attenuates vasopressin-stimulated osmotic water reabsorption in isolated microperfused rabbit collecting ducts. Subsequent cell culture studies showed that PGE2 counteracts AVP action by enhancing AQP2 endocytotic uptake from the apical plasma membrane (33). On the other hand, recent studies showed that inhibition of COX by indomethacin reduced renal AQP2 expression in rats, implying that PGs may actually help maintain AQP2 levels (20).

In addition to the well-known renal effects of PGs on urine concentration, other evidence suggests that COX-derived products could influence this process by acting at central locales. Early studies showed that AVP release was stimulated by PGE2 in the rat neurohypophysis (32). Ishikawa et al. (17) showed a reduced release of AVP in response to either hyperosmotic exposure or ANG II in guinea pig hypothalameurohypophyseal cultures following treatment with indomethacin. Finally, an intracerebroventricular (icv) injection of PGE2 in rabbits increased the concentrations of plasma AVP (12). Thus, the accumulated evidence supports a role for PGs, including PGE2 in mediating both central AVP release as well as more distal renal urine concentrating mechanisms. However, the specific receptors involved with such actions remain unidentified.

PGE2 exerts its physiological influence by interacting with four E-prostanoid (EP) receptor subtypes designated EP1, EP2, EP3, and EP4 (10). These G protein-coupled receptors are distinguished by unique ligand binding profiles, signal transduction pathways, and exhibit widespread expression including the kidney, vasculature, and nervous system (6, 8, 9). With respect to PGE2s ability to modulate urine concentrating mechanisms, previous studies focused on the EP1 receptor subtype as it is highly expressed in the principal cells of the collecting duct. Signaling through this subtype involves a G1i-coupled mechanism which limits AVP-mediated cAMP production required for water transport (15). More recent studies have suggested that the EP3 subtype carries out such putative diuretic effects by activating Rho kinase and F-actin polymerization (31). However, mice deficient for the EP3 receptor can adequately concentrate their urine in response to water deprivation (WD) suggesting that this subtype may not be a prominent player (11). On the other hand, the EP1 receptor is a reasonable candidate for modulating urine concentrating mechanisms since it is expressed along the AVP-responsive collect-
ing duct and in the PVN and SON of the hypothalamus where AVP is synthesized (4, 23). EP1 receptors can elicit phosphatidylinositol turnover and intracellular Ca2+ elevation through a Gq protein. The requirement of the EP1 receptor subtype in urine concentrating mechanisms has not been investigated.

In the present study, we propose a novel role for the EP1 receptor subtype in the setting of body fluid control. Despite an abundant expression of EP1 receptors along the collecting duct, we report that water-deprived mice lacking the EP1 receptor inadequately concentrate their urine due to a defect in central AVP production.

METHODS

Construction of EP1 targeting vector and generation of EP1 targeting mice using the “Hit & Run” strategy. The strategy for generating EP1 gene-targeted mice is described in detail in another concurrent manuscript. Briefly, mouse genomic clones containing the EP1 gene locus were obtained by screening a 129SV/Jola genomic P1 library (Genome Systems, St. Louis, MO) with a 233-bp murine EP1 cDNA fragment from the coding region of exon 2. A 5,067-bp fragment containing all three exons of the EP1 gene was subcloned into pBluescript (Stratagene) at HinIII and XbaI sites. An 895-bp fragment was then amplified from this clone, and using PCR-based mutagenesis its sequence was altered. The mutation introduces a stop codon in the coding region of the EP1 receptor [C795T changes an in-frame CGA (R242) to TGA thereby halting translation at the beginning of the third cytoplasmic loop of the EP1 receptor, resulting in a nonfunctional receptor. The 5,067-bp mutant EP1 fragment was then subcloned into the plasmid pKOScrambler NTKV (Lexigen, Woodlands, TX) which contains neo and TK selection cassettes.

Targeting the murine EP1 gene was accomplished by two steps using the strategy for “Hit & Run.” First, an EP1 gene targeting vector was linearized by BstEII at a unique site between exon 1 and 2, before electroporation of TL-1 embryonic stem (ES) cells. The linearized mutant gene inserts along with the neo and TK gene were introduced into ES cells, allowing homologous recombination via a single crossover event which results in a direct repeat tandem duplication of the EP1 gene. Insertion events were selected by G418 resistance and resistant clones were isolated and confirmed by Southern blotting. Since the direct repeat of the EP1 gene is unstable, spontaneous deletion of one allele arising via single, reciprocal intrachromosomal recombination occurs between the direct repeats, leading to deletion of the selectable cassettes, the plasmid backbone, and one repeat of the targeted loci. Targeted ES cells bearing the mutant allele were injected into blastocysts and introduced into a foster mother according to standard procedures carried out at the Vanderbilt University Transgenic Core. Founder lines were obtained and mice were backcrossed to congenenity with C57BL/6 mice for 10 generations.

WD and water-loading experiments. For determination of drinking water intake and urine volume, adult mice (8–12 wk old) were transferred to metabolic cages (Nalge Nunc International) with free access to food and water for 3 days to acclimate them. Subsequently, urine volume and drinking water consumption were monitored for 5 consecutive days. For WD experiments, mice were deprived of access to drinking water by removing the water bottles and administered either vehicle (100 μl sc normal saline) or [deamino-Cys1,d-Arg8]-vasopressin (DDAVP; 1.0 μg/kg sc, 100 μl) 24 h before death. In each experiment, urine spot samples were obtained at 4 and 24 h following removal of water bottles. Body weights were recorded before and after WD. For some experiments, a small volume of blood was drawn from the right hindpaw, collected in heparinized tubes, and centrifuged at 1,500 g to isolate plasma. For water-loading experiments, mice were administered sterile water (0.5 ml/10 g ip) and concurrently injected with either vehicle (100 μl sc normal saline) or DDAVP (1.0 μg/kg sc, 100 μl). Spot urine samples were obtained at 0, 6, and 24 h following water loading. Urine and plasma osmolality were determined by freezing point depression (Advanced Instruments model 3MO plus). AVP peptide levels were assayed by ELISA (R&D Systems) according to the manufacturer’s instructions by employing a 1:20 dilution of urine obtained from either control or 24-h WD mice. Results were corrected for urine creatinine (Cr) content determined by picric acid assay (Sigma).

Medullary osmolality determination. Mice were either given free access to water (control) or water deprived for 24 h. Kidneys were processed according to a method developed by Schmidt-Nielsen et al. (28a) as modified by Fenton et al. (10a). The kidneys were excised and the inner medullas were rapidly dissected, blotted onto Whatman 3M filter paper, and placed into preweighed microfuge tubes. Samples were weighed immediately and subsequently dried in an oven over a desiccant at 60°C for 8 h. After reweighing, 25 μl of deionized, distilled water were added to each tube and immersed in a boiling water bath for 3 min. Tubes were then centrifuged briefly and incubated at 4°C for 24 h to allow for diffusion. After centrifugation for 5 min at 8,000 g, the supernatant was analyzed for osmolality by freezing point depression and corrected for the original water content of the sample.

Quantitative RT-PCR. Groups of WT and EP1 knockout mice either given free access to water or water deprived for 24 h were subsequently euthanized by cervical dislocation. Brains and kidneys were rapidly dissected. Brains were placed in a mouse brain matrix (Harvard Apparatus, Holliston, MA) and 2-mm coronal slices were collected from the rostral to caudal extent of the forebrain. The hypothalamic region surrounding the ventral portion of the third ventricle was excised from two consecutive brain slices and tissue was placed in a 1.5-ml Eppendorf tube containing 200 μl of RNeasy (QUI, GIBCO) for future real-time RT-PCR analysis.

Total RNA was extracted from the tissues and subjected to DNase treatment (QIAGEN, Valencia, CA). AQP2 mRNA levels were determined by real-time RT-PCR using a two-step RT-PCR Syber Green detection approach. PreproAVP (vasopressin-neurophysin II GI:202341) mRNA levels were assayed using a TagMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA). Reactions were carried out using 50 ng of total RNA under the following conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, using an ABI Prism 7000 Sequence Detection System. Primers and TaqMan probe for preproAVP: forward primer 5′-cgc tct ccg ctt gtc tcc 3′, reverse primer 5′-tgca cgg act gaa gta gca-3′ and probe 5′-conc ggct cct ctc ctc cgc-c TAMRA. Primers for AQP2: forward primer 5′-atc ttt gcc acc gat ga-3′, reverse primer 5′-gct ggt gaa atg cct cac cag-3′. Relative values were derived by interpolation from a standard curve and were normalized to GAPDH mRNA levels in each sample as determined by either a TaqMan Rodent GAPDH Control Reagent kit or with specific mouse GAPDH primers when SYBR green detection was employed (Applied Biosystems).

Immunohistochemistry of tissue sections. Kidneys for immunohistochemical analysis were fixed in 4% paraformaldehyde, dehydrated through a gradient of alcohols, and embedded in paraffin wax. Embedded tissues were sectioned at 7 μm and mounted onto glass slides. Slides were dehydrated and washed three times in PBS and blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h at RT. Following three washings in PBS, the sections were incubated with the anti-rabbit AQP2 primary antibody (Santa Cruz Biotechnology) 1:1,000 for 1 h at RT, washed three times, and incubated with the secondary antibody (anti-rabbit) for 1 h at RT. Sections were washed three times in PBS and processed for Vector ABC3/3′-diaminobenzidine staining according to the manufacturer’s instructions (Vector Laboratories). Sections were visualized by high-power light microscopy.

Statistics. The values are presented as means ± SE. Statistical comparisons between two groups were performed using the unpaired Student’s t-test. Comparisons between three or more groups were
RESULTS

**Urinary concentrating ability of EP<sup>1</sup>−/− mice.** Overall, adult EP<sup>1</sup>−/− mice (8–12 wk of age) backcrossed for 10 generations onto a congenic C57Bl/6J background were healthy, fertile, and similar in size and weight to their WT counterparts (females: WT, 20.1 ± 1.0 g; EP<sup>1</sup>−/−, 20.0 ± 0.3 g, not significant; males: WT, 23.5 ± 0.4 g; EP<sup>1</sup>−/−, 24.2 ± 0.5 g, not significant). However, as shown in Fig. 1, daily urine output (WT, 0.05 ± 0.01 ml·g body wt<sup>−1·day<sup>−1</sup>; EP<sup>1</sup>−/−, 0.07 ± 0.01, P < 0.03) and water consumption (WT, 0.24 ± 0.01 ml·g body wt<sup>−1·day<sup>−1</sup>; EP<sup>1</sup>−/−, 0.31 ± 0.03, not significant) were slightly greater in EP<sup>1</sup>−/− mice compared with WT mice. Although a lower baseline urinary osmolality was observed for the EP<sup>1</sup>−/− mice (WT, 2.166 ± 153 mosmol/kg H<sub>2</sub>O vs. EP<sup>1</sup>−/−, 1.954 ± 139 mosmol/kg H<sub>2</sub>O, P = 0.3), it was not statistically significant. To assess the near-maximal urine concentrating ability of EP<sup>1</sup>−/− mice, healthy littermates were water deprived (WD) for 24 h. As shown in Fig. 2A, urinary concentrating ability rose significantly for WT mice, but not for EP<sup>1</sup>−/− mice, as early as 4 h following the initiation of WD (WD WT, 3.328 ± 99 mosmol/kg H<sub>2</sub>O vs. WD EP<sup>1</sup>−/−, 2.000 ± 180 mosmol/kg H<sub>2</sub>O, *P < 0.01). Furthermore, urine osmolalities for WD EP<sup>1</sup>−/− did not reach levels achieved by WT WD mice even after 24 h of WD (Fig. 2A, WD WT: 3.880 ± 170 mosmol/kg H<sub>2</sub>O vs. WD EP<sup>1</sup>−/−: 2.789 ± 170 mosmol/kg H<sub>2</sub>O, *P < 0.05). Consistent with a urine concentrating defect, EP<sup>1</sup>−/− plasma osmolality rose significantly higher than that of WT mice following WD (WD WT, 291 ± 7 mosmol/kg H<sub>2</sub>O vs. WD EP<sup>1</sup>−/−, 321 ± 4 mosmol/kg H<sub>2</sub>O, **P < 0.01). Over this short time period, body weights decreased equally among WD WT and WD EP<sup>1</sup>−/− mice (WD WT: −9.5 ± 0.5% body wt vs. WD EP<sup>1</sup>−/−: −8.0 ± 0.5% body wt).

Fig. 1. Baseline urine output, water consumption, and body wts for wild-type (WT) and EP<sup>1</sup>−/− mice. Male and female mice (8–12 wk old) were housed in metabolic diuresis cages for 3 days before measurements. Mice were allowed free access to food and water throughout the study. Urine output (ml·25 g body wt<sup>−1·day<sup>−1</sup>; A), water consumption (ml·25 g body wt<sup>−1·day<sup>−1</sup>; B), and body wt (g; C) were measured daily. The values presented are the means derived from 5 consecutive days using 6 mice per group (n = 6). *P < 0.03 vs. WT. The trends in urine output and water consumption were similar in both male and female EP<sup>1</sup>−/− mice.

Fig. 2. Urine concentrating abilities of WT and EP<sup>1</sup>−/− mice. A: water deprivation (WD) was initiated by removal of water bottles from cages of WT and EP<sup>1</sup>−/− mice (ages 8–12 wk). Spot urine samples were obtained at 0-, 4-, and 24-h time points. Urine osmolality was assayed by freezing point depression. The experiment was repeated 3 times and the data are means ± SE of n = 13 mice in total. *P < 0.01 vs. WD EP<sup>1</sup>−/− 4 h. **P < 0.05 vs. WD EP<sup>1</sup>−/− 24 h. B: plasma osmolality was measured in mice before and after 24 h WD. The data are means ± SE, n = 7 mice. *P < 0.01 vs. WT control. **P < 0.01 vs. WT WD and EP<sup>1</sup>−/− control.
Medullary interstitial osmolality in WD EP₁⁻/⁻ mice. Urine concentrating mechanisms rely, in part, on the maintenance of an osmolar gradient across the tubular lumen and into the medullary interstitium. To identify the mechanism underlying the urine concentrating defect, renal medullary osmolalities were determined in WD animals. As shown in Fig. 3, renal medullary osmolality dropped significantly in the EP₁⁻/⁻ mice compared with WT counterparts following WD (EP₁⁻/⁻ Δ=−466 mosmol/kgH₂O), indicating a possible defect in the regulation of the medullary osmolality gradient. The decline observed for the WD WT mice (Δ=−135 mosmol/kgH₂O) was not statistically significant compared with WT controls.

AQP2 expression in WD EP₁⁻/⁻ mice. Urine concentration also depends, in part, on the expression, translocation, and activity of AQP2 water channels in the principal cells of the collecting duct. Such transcriptional and posttranslational control of AQP2 can be subject, in part, to AVP-mediated signaling cascades (24) which are, in turn, thought to be modulated by EP receptor signaling (13, 15). Other studies carried out in primary cultures of rat IMCD cells show that signal transduction via the EP₁ receptor opposes AVP-induced AQP2 translocation to the plasma membrane (31). We investigated whether the absence of the EP₁ receptor affects AQP2 expression and translocation in response to WD. As shown in Fig. 4A, baseline AQP2 mRNA and protein expression were similar for WT and EP₁⁻/⁻ mice permitted free access to water (WT, 1.0 ± 0.1 relative units; EP₁⁻/⁻, 0.9 ± 0.1 relative units, not significant). WD for 24 h induced a significant yet equivalent upregulation of renal AQP2 in WT and EP₁⁻/⁻ mice (WT, 2.1 ± 0.3 relative units; WD EP₁⁻/⁻, 1.8 ± 0.3 relative units, P < 0.05 vs. respective water-fed controls; not significant vs. WD counterparts). Immunohistochemical analyses also revealed similar expression of AQP2 protein in WT and EP₁⁻/⁻ mice along the collecting duct from cortex to papilla (Fig. 4B, top; cortex shown). WD conferred a noticeable shift of AQP2 to the apical membranes in many of the principal cells from WT and EP₁⁻/⁻ mice (Fig. 4B, middle). Last, the combination of WD and DDAVP administration markedly induced AQP2 translocation to apical membranes along the collecting duct in WT and EP₁⁻/⁻ mice (Fig. 4B, bottom).

Reduced AVP levels in WD EP₁⁻/⁻ mice. AVP is generated in the magnocellular neurons of the hypothalamic PVN and SON. Recent studies localized the EP₁ receptor to the PVN and SON (23). We therefore hypothesized that AVP production might be compromised in the absence of the EP₁ receptor. Accordingly, preproAVP mRNA levels were measured in microdissected hypothalamic regions from WD mice by quantitative RT-PCR. As shown in Fig. 5A, a TaqMan primer/probe set directed against preproAVP mRNA detected significant induction of AVP mRNA expression in hypothalamic tissue of WT mice following WD for 24 h (WT, 0.4 ± 0.2 relative units vs. WD WT, 1.7 ± 0.4 relative units, P < 0.01). However, this effect was blunted in WD EP₁⁻/⁻ mice (EP₁⁻/⁻, 0.3 ± 0.2 relative units vs. WD EP₁⁻/⁻, 0.6 ± 0.1 relative units, not significant). Urinary AVP peptide levels were also determined for control and WD WT and EP₁⁻/⁻ mice by ELISA. As shown in Fig. 5B, WD for 24 h yielded a substantial increase of urinary AVP in WT mice (WT, 4.9 ± 0.2 pg/mg Cr vs. WD WT, 16.1 ± 1.5 pg/mg Cr, P < 0.001). In contrast, this manipulation produced a more modest response by EP₁⁻/⁻ mice that was statistically significant from WT mice (EP₁⁻/⁻, 4.2 ± 0.3 pg/mg Cr vs. WD EP₁⁻/⁻, 11.2 ± 0.9 pg/mg Cr, P < 0.001; P < 0.01 vs. WD WT). Administration of a supraphysiological dose of DDAVP (1.0 µg/kg sc), a highly selective V₂ receptor agonist, restored a significant proportion of the concentrating ability in WD EP₁⁻/⁻ mice, with the change in urine osmolality increasing from Δ+464 ± 243 mosmol/kgH₂O to Δ+1,624 ± 140 mosmol/kgH₂O (Fig. 6A). However, these mice were slightly less responsive than the DDAVP-treated WT mice (WD WT, Δ+1,520 ± 122 mosmol/kgH₂O vs. WD WT + DDAVP, Δ+2,464 ± 146 mosmol/kgH₂O). To better understand whether the defect in urine concentration possessed a renal component, mice were water loaded to suppress endogenous AVP levels. Mice were simultaneously administered water (0.5 ml/10 g ip) with either saline or DDAVP (1.0 µg/kg sc). As shown in Fig. 6B, urine osmolalities measured 6 h post water loading revealed that WT and EP₁⁻/⁻ mice concentrated their urine similarly in response to DDAVP (water-loaded WT + DDAVP increasing from 1,420 ± 253 to 2,540 ± 232 mosmol/kgH₂O, vs. water-loaded EP₁⁻/⁻ rising from 1,772 ± 311 to 3,036 ± 174 mosmol/kgH₂O). Water loading without DDAVP administration yielded a diluted urine, as expected (water-loaded WT, 815 ± 62 mosmol/kgH₂O, vs. water-loaded EP₁⁻/⁻, 396 ± 101 mosmol/kgH₂O). Urine osmolalities for all groups of mice returned to baseline values after 24 h.

DISCUSSION

Several lines of evidence suggest that PGE₂ contributes to homeostatic volume control by modulating urine concentrating mechanisms. However, the specific EP receptor(s) and their functional locales accounting for these effects have not been completely identified. In the present study, we investigated whether the PGE₂ EP₁ receptor subtype participates in urine concentration as it is expressed in the AVP-producing hypothalamus and along the AVP-responsive collecting duct. Our data reveal a defect in central AVP synthesis for mice lacking the EP₁ subtype following acute WD. The blunted hypothalamic AVP production in EP₁-null animals suggests that this receptor is a pivotal player in the response to hyperosmolality. Moreover, our findings suggest that the EP₁ receptor acts upstream of AVP production, likely along paraventricular
and/or supraoptic inputs within the hypothalamus. AVP is initially produced as a larger preproAVP protein in the hypothalamus. Following proteolytic processing by neurophysin, the mature AVP peptide is stored in the posterior pituitary where it awaits appropriate physiological cues before being secreted into the venous flow. Studies by Arima et al. (3) showed that pituitary stores of AVP are quickly depleted following WD thereby requiring induction of mRNA synthesis to sustain downstream urine concentrating mechanisms. Such stores likely account for the modest rise in urinary AVP in EP1/−/− mice observed in the present study. However, we suspect that while these quantities are rapidly exhausted to similar extents in WT and EP1/−/− mice, they are replenished at a slower rate in the latter animals. The net result is that the

Fig. 4. Renal AQP2 in water-deprived WT and EP1/−/− mice. Water bottles were removed from the cages of WT and EP1/−/− mice (ages 8–12 wk) and after 24 h the animals were killed along with a group of control animals given free access to water. Another group of WT and EP1/−/− mice were administered DDAVP (1.0 μg/kg sc) at the time of water bottle removal. The kidneys were removed and processed for either RNA isolation or immunohistochemistry as described in Methods: A: real-time RT-PCR of renal AQP2 mRNA. DNase-treated mRNA was reverse-transcribed and subsequently assayed for AQP2 content using a primer set in conjunction with SYBR green-based Quantitect PCR detection kit (Qiagen). Data are normalized to GAPDH content and relative values are derived from standard curves constructed for each target. The data are means ± SE of n = 5 mice. *P < 0.05 vs. respective nonwater-deprived controls. B: AQP2 immunohistochemistry of renal sections (×400 magnification). Whole kidney sections (7-μm thickness) derived from control, water-deprived, and DDAVP-treated water-deprived mice were subjected to immunohistochemical detection with a goat anti-AQP2 polyclonal antibody (1:1,000 dilution). Representative images of cortex AQP2 labeling obtained from groups of at least 5 mice are shown. Similar results were seen in outer/inner medullary regions. Arrowheads denote substantial apical membrane localization of AQP2.
EP1-null kidney receives a suboptimal AVP quantity, thereby limiting urine concentration.

The intrarenal role of the EP1 receptor is only partially characterized. In the kidney, this Gq-coupled subtype is found in the mesangial cells and podocytes, where it may modulate certain maladaptive aspects of diabetic nephropathy such as glomerular hypertrophy, matrix expansion, proteinuria, and TGF-β production (22). Along the nephron, the EP1 receptor is abundantly expressed in the principal cells of the collecting duct, where it is thought to offset the buffering influence of EP3 receptors upon the anti-diuretic actions of AVP (31). In vitro studies showed that PGE2 can inhibit sodium transport in the isolated, perfused rabbit collecting duct by a mechanism coupled to the release of Ca2+ from intracellular stores, consistent with EP1-mediated signal transduction (14). In contrast, work by Coffman and colleagues (29) supports an anti-natriuretic role for the EP1 receptor. They observed a reduction in systolic blood pressure and a rise in plasma renin activity in EP1 knockout mice fed a low-sodium diet. However, our data argues against a substantial role for this subtype in distal collecting duct Na+ handling since EP1−/− mice exhibit appropriate anti-natriuresis or natriuresis when placed on either sodium-deficient or high-sodium diets, respectively (data not shown). This result is consistent with findings obtained in rats where EP1 receptor levels were unaffected by dietary sodium manipulation (18). However, our interpretation must be tempered by the possibility that other receptor pathways may offer redundant mechanisms to compensate for loss of the EP1 receptor.

PGE2 tempers the actions of AVP in isolated, perfused rabbit collecting duct, yet when added alone, this eicosanoid promotes transepithelial water flow (15). Subsequent work suggested that PGE2 exerts such anti-diuretic effects via cAMP-coupled EP4 receptors (28), whereas PGE2’s ability to reduce AVP-induced cAMP production implies EP1/3 signaling (26). However, the mechanism underlying these effects may be more complex, as demonstrated by the recent work of Klussman and colleagues (31). Their data suggest that EP3 receptors antagonize the inhibitory effects of the EP3 receptor on AQ22 translocation in rat inner medullary collecting duct cells through effects on Rho GTPase and the actin cytoskeleton. According to such a model, the urinary concentrating defect observed presently in EP1−/− mice would arise due to an unmasking of the EP3 subtype, yielding significant attenuation of EP1−/− mice.

A: hypothalamic AVP mRNA induction in WT and EP1−/− water-deprived mice. Water bottlers were removed from the cages of WT and EP1−/− mice (ages 8–12 wk) and after 24 h the animals were killed along with a group of control animals with free access to water. Hypothalamic regions were excised and the total RNA was extracted as described in METHODS. A TaqMan primer/probe set directed against preproAVP was used to quantify AVP induction. Data are normalized to GAPDH content and relative values were derived from standard curves constructed for each target. The data are means ± SE of n = 5 mice. *P < 0.01 vs. WT control.

B: urinary AVP content in water-deprived WT and EP1−/− mice. Mice were subjected to water deprivation for 24 h and urine AVP content was assayed by ELISA. Values presented are corrected for urine creatinine content. **P < 0.001 vs. WT control. *P < 0.01 vs. EP1−/− control and water-deprived WT.

Fig. 5. AVP synthesis in water-deprived WT and EP1−/− mice. A: hypothalamic AVP mRNA induction in WT and EP1−/− water-deprived mice. Water bottles were removed from the cages of WT and EP1−/− mice (ages 8–12 wk) and after 24 h the animals were killed along with a group of control animals with free access to water. Hypothalamic regions were excised and the total RNA was extracted as described in METHODS. A TaqMan primer/probe set directed against preproAVP was used to quantify AVP induction. Data are normalized to GAPDH content and relative values were derived from standard curves constructed for each target. The data are means ± SE of n = 5 mice. *P < 0.01 vs. WT control. B: urinary AVP content in water-deprived WT and EP1−/− mice. Mice were subjected to water deprivation for 24 h and urine AVP content was assayed by ELISA. Values presented are corrected for urine creatinine content. **P < 0.001 vs. WT control. *P < 0.01 vs. EP1−/− control and water-deprived WT.

Fig. 6. DDAVP responsiveness of water-deprived and water-loaded WT and EP1−/− mice. A: DDAVP partially restores urinary concentrating ability of EP1−/− water-deprived mice. Mice were administered DDAVP (1.0 µg/kg sc) or saline at the time of water loading (0.5 ml/10 g body wt ip) and the urine osmolalities were determined at 0, 6, and 24 h. The data are presented as the change (Δ) in urine osmolality of WT and EP1−/− mice exhibit appropriate anti-natriuresis or natriuresis when placed on either sodium-deficient or high-sodium diets, respectively (data not shown). This result is consistent with findings obtained in rats where EP1 receptor levels were unaffected by dietary sodium manipulation (18). However, our interpretation must be tempered by the possibility that other receptor pathways may offer redundant mechanisms to compensate for loss of the EP1 receptor. PGE2 tempers the actions of AVP in isolated, perfused rabbit collecting duct, yet when added alone, this eicosanoid promotes transepithelial water flow (15). Subsequent work suggested that PGE2 exerts such anti-diuretic effects via cAMP-coupled EP4 receptors (28), whereas PGE2’s ability to reduce AVP-induced cAMP production implies EP1/3 signaling (26). However, the mechanism underlying these effects may be more complex, as demonstrated by the recent work of Klussman and colleagues (31). Their data suggest that EP3 receptors antagonize the inhibitory effects of the EP3 receptor on AQ22 translocation in rat inner medullary collecting duct cells through effects on Rho GTPase and the actin cytoskeleton. According to such a model, the urinary concentrating defect observed presently in EP1−/− mice would arise due to an unmasking of the EP3 subtype, yielding significant attenuation of EP1−/− mice.

A: hypothalamic AVP mRNA induction in WT and EP1−/− water-deprived mice. Water bottles were removed from the cages of WT and EP1−/− mice (ages 8–12 wk) and after 24 h the animals were killed along with a group of control animals with free access to water. Hypothalamic regions were excised and the total RNA was extracted as described in METHODS. A TaqMan primer/probe set directed against preproAVP was used to quantify AVP induction. Data are normalized to GAPDH content and relative values were derived from standard curves constructed for each target. The data are means ± SE of n = 5 mice. *P < 0.01 vs. WT control. B: urinary AVP content in water-deprived WT and EP1−/− mice. Mice were subjected to water deprivation for 24 h and urine AVP content was assayed by ELISA. Values presented are corrected for urine creatinine content. **P < 0.001 vs. WT control. *P < 0.01 vs. EP1−/− control and water-deprived WT.

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of AVP-mediated water reabsorption. However, in vivo evidence for direct involvement of EP1 receptors remains elusive. Coffman and colleagues showed that EP1-/- mice do not “over concentrate” their urine in response to WD, although inhibition of endogenous PGE2 production by indomethacin yields an elevation of urinary osmolality in WT but not in EP1-/- mice (11). Such findings suggest that the EP1 receptor may underlie increased urine concentration encountered with rodents and humans following NSAID administration (5). Our in vivo data with EP1-/- mice suggest that collecting duct EP1 receptors may influence only a minor portion of AVP-induced water flux in the context of WD. Much of the urine concentrating ability of WD EP1-null animals was restored by DDAVP administration. Furthermore, WD combined with DDAVP induced a qualitatively similar AQP2 translocation from a subapical to apical plasma membrane along the collecting duct in WT and EP1-/- mice. When endogenous AVP levels were suppressed by acute water loading, WT and EP1-/- mice were equally responsive to DDAVP-induced urine concentration, once again suggesting that AQP2-based urine concentrating mechanisms are intact despite the absence of functional EP1 receptors along the collecting duct. However, the acute reduction of medullary osmolality observed in EP1-/- mice following WD may also have contributed to the urine concentrating defect. Whether reduced AVP levels in response to WD might account for this drop in medullary osmolality in EP1-/- mice is unclear, and warrants further investigation. Furthermore, it was surprising that WD failed to increase medullary osmolality in WT mice. Although values trended toward a reduction in medullary osmolality, they did not achieve statistical significance. It is probable that the mouse can sustain its renal medullary osmolality for the 24-h period of WD and that an increase would likely follow at later time points. On the other hand, the result may be due to methodological limitations. Inasmuch as our data suggest that collecting duct EP1 receptors play but a minor role in the modulation of AVP action within this nephron segment, we cannot rule out possibility that adaptive mechanisms in the collecting duct may have arisen in the absence of the EP1 receptor.

Our work therefore suggests that the EP1 receptor subtype largely contributes to urine concentrating mechanisms; not through its actions along the collecting duct, but through its expression in the hypothalamus. This hypothesis is consistent with recent work by two groups who localized the EP1 receptor to the hypothalamus. In situ hybridization studies detected EP1 receptor mRNA in the supraoptic nuclei (4) while others employed an EP1 antiserum to verify expression in the central nucleus of amygdala and at synapses of corticotrophin-releasing hormone containing neurons of the PVN (23). These structures each contribute to the synthesis of AVP in response to a variety of physiological and pathophysiological stimuli. In fact, the involvement of AQP2 in mediating AVP synthesis was suggested by a number of reports. For example, icv administration of NSAIDs to rats can blunt AVP synthesis in response to hypertonic saline (7), acute WD, or injection of bacterial endotoxin (21). Furthermore, icv injection of PGE2 in rats increased plasma AVP levels in some studies (12, 27) while not in others (21). Until now, the identity of the specific prostanoid receptor underlying these effects remained unclear. We suggest that such NSAID-induced abrogation of PGE2 synthesis suppresses EP1 receptor activation in the PVN/SON, thereby limiting AVP production and restricting renal urine concentrating mechanisms. Accordingly, our data show that AVP mRNA induction is blunted in EP1-/- mice in response to acute WD, while baseline production and circulating levels of AVP are sufficient to avoid a central diabetes insipidus phenotype in these mice. This finding is likewise consistent with studies in rats showing that while indomethacin treatment blunts AVP production in response to icv infusion of hypertonic saline, it does not affect baseline AVP levels (17).

A role for centrally expressed EP1 receptors was recently suggested by others. Adrenocorticotropic hormone (ACTH) is produced in the PVN, is stored in the anterior pituitary, and is released in response to inflammatory stress. Narumyia and colleagues (23) demonstrated that endotoxin-treated EP1-/- mice exhibited an impaired ACTH response coupled with reduced c-fos induction. One hour after receiving LPS injections, EP1-/- mice were unable to significantly elevate plasma ACTH levels in contrast to their WT counterparts. Taken together, it would therefore appear that centrally produced PGE2 acting via the EP1 receptor subtype in the hypothalamus plays a significant role in mediating the body’s response to various physiological and pathophysiological stresses. The involvement of the EP1 subtype in urine concentrating mechanisms is of particular interest since recent studies are driving the development of EP1-selective ligands as potential anti-cancer agents (16, 19). Furthermore, such reagents have proved therapeutic for rats with glomerulopathies induced by diabetes or hypertension (22, 30). In light of the current study, the application of EP1 receptor-based therapeutics may therefore require careful consideration of the potential effects on urine concentrating mechanisms.

**NOTE ADDED IN PROOF**

On p. F869 in methods, under the subheading Medullary osmolality determination, a protocol for determining renal medullary osmolality contains text which originally appeared in a 2004 article by R. A. Fenton et al. in the Proceedings of the National Academy of Sciences. Regrettably, this article was not referenced in our Articles in Press manuscript, and a reference that was listed [Schmidt-Nielsen et al. (4)], corresponding to a 1983 article by B. Schmidt-Nielsen et al. in the American Journal of Physiol-Renal Physiol, was likewise absent from our reference list. With sincere apologies to the editors of the American Journal of Physiology-Renal Physiol, the Proceedings of the National Academy of Sciences, and, importantly, to the authors of these articles, we have amended and properly referenced the section in METHODS, which will appear in the final print version of the article.

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**REFERENCES**


