Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2012 October ; 99(0): 45–50. doi:10.1016/j.prostaglandins.2012.07.001.

Tumor necrosis factor-alpha induces renal cyclooxygenase-2 expression in response to hypercalcemia

Sailaja Battula, Shoujin Hao, Paulina L. Pedraza, Charles T. Stier, and Nicholas R. Ferreri
Department of Pharmacology, New York Medical College, Valhalla, NY 10595

Abstract

The effect of tumor necrosis factor-alpha (TNF) on cyclooxygenase-2 (COX-2) expression in the renal outer medulla (OM) was determined in a model of dihydrotachysterol (DHT)-induced hypercalcemia. Increases in serum calcium and water intake were observed during ingestion of a DHT-containing diet in both wild type (WT) and TNF deficient mice (TNF^{-/-}). Polyuria and a decrease in body weight were observed in response to DHT treatment in WT and TNF^{-/-} mice. A transient elevation in urinary TNF was observed in WT mice treated with DHT. Moreover, increased urinary levels of prostaglandin E₂ (PGE₂) and a corresponding increase in COX-2 expression in the OM were observed in WT mice fed DHT. Increased COX-2 expression was not observed in TNF^{-/-} mice fed DHT, and the characteristics of PGE₂ synthesis were distinct from those in WT mice. This study demonstrates that COX-2 expression in the OM, secondary to hypercalcemia, is TNF-dependent.

Keywords

COX-2; thick ascending limb; TNF; hypercalcemia; calcium-sensing receptor

1. Introduction

We recently demonstrated that tumor necrosis factor-alpha (TNF) gene deletion is linked to an increase in Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2) protein expression and activity in the medullary thick ascending limb of Henle's loop [1]. Previous work from our laboratory demonstrated that calcium sensing receptor (CaR) activation increases TNF production and cyclooxygenase (COX)-2-mediated PGE₂ synthesis, which contributes to inhibition of sodium transport in cultured medullary thick ascending limb (mTAL) cells [2–4]. These findings are consistent with *in vivo* studies showing that TNF increases urine volume and the fractional excretion of sodium in mice [5, 6]. Moreover, CaR stimulation increases nuclear factor of activated T cells (NFAT5)-dependent TNF production, and inhibits apical chloride uptake mediated by NKCC2 in primary cultures of mouse mTAL cells [7, 8]. CaR plays a critical role in the regulation of calcium homeostasis. For instance, stimulation of this receptor by a mechanism that is independent of its effects on parathyroid hormone promotes Ca²⁺ excretion in response to hypercalcemia [9–12].

© 2012 Elsevier Inc. All rights reserved.

To whom correspondence and reprint requests should be addressed: Dr. Nicholas R. Ferreri, Department of Pharmacology, New York Medical College, Valhalla, NY 10595, Phone: 914-594-4079, FAX: 914-347-4956, nick_ferreri@nymc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The TAL is responsible for approximately 25% of NaCl uptake in the kidney and plays a role in the long-term regulation of blood pressure and extracellular fluid volume. Variations in NaCl reabsorption in this nephron segment, for example, may increase susceptibility to various cardiovascular diseases [13]. The TAL is capable of metabolizing arachidonic acid via the COX and cytochrome P450 pathways, and the products formed have been shown to inhibit ion transport in the TAL [14, 15]. COX-2 is constitutively expressed along the TAL and is upregulated *in vivo* by stimuli such as angiotensin converting enzyme inhibitors, changes in salt intake, adrenalectomy, and diabetes [16–20]. The role of TNF as an *in vivo* regulator of COX-2 expression in the kidney has not been addressed.

Induction of hypercalcemia in rats is associated with an elevation in COX-2 and phospholipase A₂ (PLA₂) expression in the cortex, outer medulla, and inner medulla of the kidney [21]. Moreover, renal PGE₂ was significantly elevated during hypercalcemia concomitant with defective NaCl reabsorption in the TAL [22]. The mechanisms that regulate renal COX-2 expression and synthesis of PGE₂ in hypercalcemia have not been fully described. Since CaR is activated in response to exogenous vitamin D [10], and CaR activation was linked to increased TNF production and subsequent stimulation of COX-2 expression in primary cultures of mTAL cells [4, 7, 10], we hypothesize that COX-2 expression in response to hypercalcemia is TNF-dependent.

2. Materials and methods

2.1. Animals

Male B6129S-*Tnfr1Gkl/J* TNF deficient mice (TNF^{-/-}) congenic on the C57BL/6J genetic background, and C57BL/6J wild-type (WT) mice were purchased from the Jackson Laboratory and maintained on standard diet and tap water *ad libitum*. Experimental procedures were conducted in accordance with institutional and international guidelines for the welfare of animals (animal welfare assurance number A3362-01, Office of Laboratory Animal Welfare, PHS, NIH). Seven-week-old mice weighing 20–22g were acclimated in metabolic cages for 3 days and fed a control diet (0.6% NaCl diet, from Harlan Teklad, WI). Hypercalcemia was induced by adding 4.2 mg dihydrotachysterol (DHT)/kg dry food to a control diet. DHT was purchased from Sigma and the DHT diet was custom made by Harlan Teklad. Food intake, water intake, body weight, and urine volume were measured daily throughout the study. Blood for the measurement of serum parameters was collected by cardiac puncture immediately after anesthesia at the time of sacrifice. Kidneys were excised and the outer medulla was dissected and snap frozen in liquid nitrogen and stored at –80°C until use.

2.2. Chemicals and reagents

The PGE₂ ELISA kit and antibody for COX-2 used for immunoblotting were purchased from Cayman (Ann Arbor, MI). Collagenase (type 1A) was from Sigma (St. Louis, MO) and polyvinylidene difluoride (PVDF) membranes were obtained from Amersham (Arlington Heights, IL). Reagents for preparation of the TNF ELISA were purchased from Pharmingen (San Diego, CA). All chemicals were of the highest grade commercially available.

2.3. Determination of serum parameters

Serum electrolytes were analyzed with an Olympus 640e Clinical Chemistry Analyzer (Antech Diagnostics, NC).

2.4. Measurement of TNF

ELISA was used to determine TNF levels in urine according to the protocol provided by the manufacturer (Pharmingen), as previously described [4].

2.5. Western blotting

Renal outer medulla (OM) was homogenized in cell lysis buffer from Cell Signaling (Catalog No.9803) for analysis of COX-2 expression. Equal amounts of OM protein lysates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Following blocking at room temperature for 1 hr with 5% skim milk, membranes were probed overnight with COX-2 antibody (1:1,000), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech). Membranes were washed, and proteins detected by enhanced chemiluminescence (ECL; Amersham).

2.6. Measurement of PGE₂

PGE₂ levels in urine were determined by ELISA (Cayman), according to the protocol provided by the manufacturer and as previously reported [2, 4].

2.7. Statistical analysis

Two-way ANOVA followed by Newman-Keuls test was used to determine differences between strains and treatment. Paired Student's *t*-test was used to determine the statistical significance between control and DHT-containing diet groups. All data are presented as mean ± SEM (standard error); *p* < 0.05 is considered statistically significant.

3. Results

3.1. Characteristics of DHT-induced hypercalcemia in WT and TNF^{-/-} mice

The influence of DHT administration on calcium homeostasis and renal function in mice has not yet been reported. Food intake and body weight decreased in mice ingesting a DHT-containing diet, similar to previous reports in rats [23, 24]. Food intake was greater in TNF^{-/-} mice compared with WT mice (Fig. 1A). However, WT and TNF^{-/-} mice showed a significant decrease in body weight starting on day 3 and continuing through day 7 of the DHT treatment (Fig. 1B). Serum concentrations of Na⁺, K⁺, Mg²⁺ and P were similar in WT and TNF^{-/-} mice given a DHT-containing diet (not shown). However, TNF^{-/-} mice had a slightly higher basal serum Ca²⁺ level compared to WT mice (Fig. 2A). DHT treatment was associated with marked elevations in serum Ca²⁺ levels in both WT and TNF^{-/-} mice with the levels remaining slightly higher in the TNF^{-/-} mice (Fig. 2A). Urinary excretion of Ca²⁺ increased in WT and TNF^{-/-} mice, peaking on day 3, and tending to remain elevated above pre-DHT levels thereafter (Fig. 2B). There was no difference in urinary Ca²⁺ excretion between WT and TNF^{-/-} mice over the 7 days of DHT treatment, except for an earlier onset of increased Ca²⁺ excretion in WT mice (day 1) vs TNF^{-/-} mice (day 2).

Urine volume was significantly elevated in response to DHT treatment on days 2 and 3 in WT and TNF^{-/-} mice compared with urine volume in mice given control diet (Fig. 3). The increase in urine volume was transient in WT and TNF^{-/-} mice, as on days 4–7 urine volume returned to values not significantly different from pre-DHT levels (Fig. 3). However, the increase in urine volume in response to DHT was significantly attenuated in TNF^{-/-} compared with WT mice (Fig. 3). The increase in urine volume was accompanied by an increase in water intake on days 2 and 3 in response to DHT intake in both WT and TNF^{-/-} mice (Fig. 4).

3.2. TNF levels increase in mice given DHT

Previous studies from our laboratory demonstrated that CaR activation by extracellular Ca²⁺ increases TNF production by mTAL cells *in vitro* [4]. In the present study, urinary TNF levels were measured in mice before and during treatment with DHT, which increased

serum calcium levels and presumably activates the CaR on the basolateral membrane the TAL [10, 11]. An increase in urinary TNF levels was observed in WT mice on days 2 and 3 of DHT treatment; approximately a four-fold increase was observed on day 3 (Fig. 5). However, this elevation was transient as levels returned to values corresponding to pre-DHT levels from days 4–7 (Fig. 5).

3.3. DHT increases COX-2 protein expression and urinary PGE₂ levels in WT mice

We previously found that TNF increases COX-2 expression and PGE₂ synthesis in primary cultures of mTAL cells [2]. Since DHT increased urinary TNF levels, the effect of DHT on COX-2 expression in OM was determined in WT and TNF^{-/-} mice, as approximately 80% of renal structures in this region are TAL tubules. COX-2 protein expression increased in the OM of WT mice fed DHT-containing diet for 1, 3 or 7 days; the highest expression was observed on day 3 (Fig. 6). The contribution of TNF to the DHT-mediated increase in COX-2 expression was then evaluated in OM from WT and TNF^{-/-} mice ingesting DHT-containing diet. Western blot analysis indicated that COX-2 expression in OM increased approximately two-fold in WT mice fed DHT for 7 days (Fig. 7A). Although there was no apparent difference in basal COX-2 expression between WT and TNF^{-/-} mice, ingestion of DHT did not induce an increase in COX-2 expression in TNF^{-/-} mice (Fig. 7B). Urinary levels of PGE₂ in WT mice were significantly elevated during days 1–6 of DHT treatment (Fig. 8A). On the other hand, a tendency for urinary PGE₂ excretion to increase that did not achieve statistical significance was observed in TNF^{-/-} mice ingesting DHT (Fig. 8B). Collectively, these data indicate that TNF contributes to DHT-mediated increases in COX-2 protein expression in the OM, and renal PGE₂ synthesis. Although not all urinary PGE₂ is derived from the TAL, the changes observed are consistent with increased COX-2 expression at that site.

4. Discussion

We demonstrated that upregulation of COX-2 expression in the renal OM is TNF-dependent in mice ingesting a diet containing DHT. The increase in COX-2 expression was observed in a model of hypercalcemia known to involve activation of the CaR [10], and is consistent with studies using primary cultures of mTAL cells demonstrating that activation of CaR increases COX-2 via a mechanism involving TNF [3, 4, 7]. A marked elevation in serum calcium was observed during ingestion of a DHT-containing diet in both WT and TNF^{-/-} mice indicating that hypercalcemia was achieved. TNF^{-/-} mice exhibited higher levels of serum calcium than WT mice, which may reflect increased appetite in the absence of TNF. Polyuria, one of the characteristic features of hypercalcemia, was observed during DHT treatment in both strains, and was greater in TNF^{-/-} compared with WT mice. A transient elevation in urinary TNF levels was observed in WT mice treated with DHT. Moreover, increased urinary levels of PGE₂ were observed, along with a corresponding increase in COX-2 expression in the OM of WT mice fed DHT. Significant increases in COX-2 and urinary PGE₂ were not observed in TNF^{-/-} mice fed DHT. Collectively, these data demonstrate that hypercalcemia increases COX-2 expression in OM via a mechanism involving TNF.

In vitro studies have shown that increased extracellular Ca²⁺, via stimulation of CaR, increases TNF gene transcription and production by mTAL cells, effects linked to increases in COX-2 expression and PGE₂ synthesis [3, 4, 7]. These observations are consistent with the notion that the production of TNF and a corresponding increase in COX-2 expression in the OM might be increased in an experimental model of hypercalcemia such as DHT-induced hypercalcemia, in which the CaR is activated [23]. Chronic daily administration of DHT to rats is a well-established model of rapidly developing hypercalcemia [22]. DHT is a synthetic vitamin D analogue that differs from vitamin D in having a hydroxyl group at

position one, and does not require renal activation but necessitates only a single hydroxylation step in the liver [22, 25]. CaR plays a role in the fine regulation of plasma calcium and controls urinary calcium excretion independently of its effects on PTH secretion and calcitonin in response to vitamin. [26, 10]. For instance, mice lacking the full-length CaR are more sensitive to the calcemic action of $1,25(\text{OH})_2 \text{D}_3$ in the setting of PTH deficiency due to enhanced gastrointestinal absorption of Ca^{2+} and decreased renal excretion of Ca^{2+} without any changes in bone-related Ca^{2+} release or calcitonin excretion [10]. In the present study, body weight decreased in both strains during ingestion of the DHT-containing diet. The reduction in body weight was slightly greater in $\text{TNF}^{-/-}$ mice compared with WT mice, but cannot be accounted for by greater fluid loss in $\text{TNF}^{-/-}$ mice, as urine volume was less in these mice than WT mice. These latter findings are consistent with previous studies showing that TNF increases urine volume in mice [5, 6]. The mechanism that offsets the apparent increase in fluid retention in TNF deficient mice is not clear. The possibility that $\text{TNF}^{-/-}$ mice display a congenital compensatory response, perhaps by increasing insensible water loss, to offset increases in water reabsorption in the absence of TNF will be addressed in future studies.

One of the characteristic features of hypercalcemia is polyuria. CaR expressed in the basolateral membranes of the TAL and apical membrane of the inner medullary collecting duct (IMCD) facilitate the sensing and response of these cell types to alterations in calcium levels. Accordingly, CaR-mediated inhibition of aquaporin-2 (AQP-2) in the IMCD region [27], may have contributed to the increase in urine volume caused by DHT. Sensing of elevated calcium levels in the blood by basolateral CaR in the TAL and inhibition of fluid reabsorption at that site occurs concomitantly with elevated Ca^{2+} concentration in the luminal fluid reaching the IMCD that activate the apical CaR and result in AQP-2 endocytosis and a blunted response for vasopressin-elicited water reabsorption [27]. Urinary TNF is a reflection of TNF produced locally within the kidney by cell types such as mesangial cells [28], glomerular cells [29], proximal tubules [30], and mTAL cells [31]. TNF production is increased by CaR activation in primary cultures of mTAL cells [3, 7], as well as mTAL tubules isolated from DHT-treated mice (data not shown). Thus, the marked reduction in the degree of polyuria in $\text{TNF}^{-/-}$ mice is most likely the consequence of the absence of TNF following CaR activation in the TAL.

The TNF-dependent increase in COX-2 expression in OM may reflect an increase in the TAL, since this region of the kidney is highly enriched in this nephron segment. Interestingly, although COX-2 expression was not elevated in outer medulla of $\text{TNF}^{-/-}$ mice treated with DHT, there was a trend for urinary PGE_2 to increase in these mice, albeit in a delayed and transient manner compared with WT mice. Although urinary PGE_2 levels reflect renal production of this prostanoid, the cell type and mechanism responsible for the TNF- and COX-2-independent increase in urinary PGE_2 in response to ingestion of DHT remains to be determined. The functional relevance of PGE_2 to mTAL function has been demonstrated [32, 33]. Moreover, prostaglandins contribute to the inhibition of NaCl reabsorption in vitamin D-induced hypercalcemia in rats [25]. Hypercalcemia is associated with defective urine concentrating ability, which is a consequence of diminished ion transport in the TAL, where Na^+ reabsorption occurs via a 2-step process [23, 34, 35]. The bumetanide/furosemide-sensitive apical NKCC2 mediates electroneutral uptake of Na^+ , K^+ and 2Cl^- with the Na^+ exiting via electrogenic exchange for K^+ driven by Na^+/K^+ -ATPase on the basolateral side. Recycling of K^+ by renal outer medullary potassium channels (ROMK) produces a lumen-positive potential that facilitates paracellular reabsorption of Ca^{2+} and increased local concentrations of Ca^{2+} that leads to activation of the CaR. It has been proposed that elevated Ca^{2+} stimulates CaR in the mTAL, which attenuates NaCl reabsorption in a “furosemide-like manner” and impairs the countercurrent mechanism, although the mechanisms are only partially defined [36], and our recent work has shown that

TNF is an inhibitor of NKCC2 [1]. The present findings suggest that COX-2 expression in the mTAL increases in concert with TNF and polyuria in WT but not TNF^{-/-} mice. Thus, TNF may contribute to the attenuation of NaCl reabsorption via COX-2-dependent PGE₂, which inhibits NKCC2, during CaR activation.

The present findings are in agreement with the effects of CaR activation in mTAL cells [2–4, 7, 8], and raise the possibility that TNF produced endogenously in response to CaR activation could be part of a mechanism that regulates TAL ion transport *in vivo*. It has been shown that PGE₂ levels were elevated in the OM in hypercalcemia and mediated inhibition of chloride reabsorption in the TAL [22]. We conclude that elevated plasma Ca²⁺ in mice ingesting DHT activates CaR in the TAL and triggers a transient increase in local TNF production that induces COX-2 expression and PGE₂ synthesis, which inhibits NKCC2 activity and NaCl reabsorption during hypercalcemia. As the increase in urine volume in response to DHT is higher in WT mice compared with TNF^{-/-} mice, TNF also may be part of the mechanism whereby CaR antagonizes the effects of vasopressin on AQP-2-mediated increases in water permeability [23, 27]. Thus, the lower urine volume in TNF^{-/-} compared with WT mice may be related to attenuated COX-2 expression and PGE₂ levels and concomitant effects at other nephron sites such as the collecting duct where PGE₂ is known to antagonize the effects of vasopressin. The results of this study suggest that increased TNF production in hypercalcemia could contribute to the polyuria and concentration defects associated with this condition.

Acknowledgments

This work was supported by NIH grants HL085439 and HL34300.

References

1. Battula S, Hao S, Pedraza PL, Stier CT, Ferreri NR. Tumor necrosis factor- α is an endogenous inhibitor of Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2) isoform A in the thick ascending limb. *Am J Physiol Renal Physiol*. 2011; 301(1):F94–100. [PubMed: 21511694]
2. Abdullah HI, Pedraza PL, McGiff JC, Ferreri NR. Calcium-sensing receptor signaling pathways in medullary thick ascending limb cells mediate COX-2-derived PGE₂ production: functional significance. *Am J Physiol Renal Physiol*. 2008; 295(4):F1082–9. [PubMed: 18684886]
3. Wang D, An SJ, Wang WH, McGiff JC, Ferreri NR. CaR-mediated COX-2 expression in primary cultured mTAL cells. *Am J Physiol Renal Physiol*. 2001; 281(4):F658–64. [PubMed: 11553512]
4. Wang D, Pedraza PL, Abdullah HI, McGiff JC, Ferreri NR. Calcium-sensing receptor-mediated TNF production in medullary thick ascending limb cells. *Am J Physiol Renal Physiol*. 2002; 283(5):F963–70. [PubMed: 12372772]
5. Shahid M, Francis J, Majid DS. Tumor necrosis factor- α induces renal vasoconstriction as well as natriuresis in mice. *Am J Physiol Renal Physiol*. 2008; 295(6):F1836–44. [PubMed: 18922887]
6. Shahid M, Francis J, Matrougui K, Majid DS. Involvement of tumor necrosis factor- α in natriuretic response to systemic infusion of nitric oxide synthase inhibitor in anesthetized mice. *Am J Physiol Renal Physiol*. 2010; 299(1):F217–24. [PubMed: 20410217]
7. Abdullah HI, Pedraza PL, Hao S, Rodland KD, McGiff JC, Ferreri NR. NFAT regulates calcium-sensing receptor-mediated TNF production. *Am J Physiol Renal Physiol*. 2006; 290(5):F1110–7. [PubMed: 16380462]
8. Hao S, Zhao H, Darzynkiewicz Z, Battula S, Ferreri NR. Expression and function of NFAT5 in medullary thick ascending limb (mTAL) cells. *Am J Physiol Renal Physiol*. 2009; 296(6):F1494–503. [PubMed: 19369291]
9. Brown EM, Chattopadhyay N, Vassilev PM, Hebert SC. The calcium-sensing receptor (CaR) permits Ca²⁺ to function as a versatile extracellular first messenger. *Recent Prog Horm Res*. 1998; 53:257–80. discussion 80–1. [PubMed: 9769711]

10. Egbuna O, Quinn S, Kantham L, Butters R, Pang J, Pollak M, et al. The full-length calcium-sensing receptor dampens the calcemic response to 1 α ,25(OH)₂ vitamin D3 in vivo independently of parathyroid hormone. *Am J Physiol Renal Physiol*. 2009; 297(3):F720–8. [PubMed: 19474191]
11. Kantham L, Quinn SJ, Egbuna OI, Baxi K, Butters R, Pang JL, et al. The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. *Am J Physiol Endocrinol Metab*. 2009; 297(4):E915–23. [PubMed: 19797241]
12. Riccardi D, Brown EM. Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol*. 2010; 298(3):F485–99. [PubMed: 19923405]
13. Jung J, Basile DP, Pratt JH. Sodium reabsorption in the thick ascending limb in relation to blood pressure: a clinical perspective. *Hypertension*. 2011; 57(5):873–9. [PubMed: 21403087]
14. Ferreri NR, An SJ, McGiff JC. Cyclooxygenase-2 expression and function in the medullary thick ascending limb. *Am J Physiol*. 1999; 277(3 Pt 2):F360–8. [PubMed: 10484519]
15. Schwartzman M, Ferreri NR, Carroll MA, Songu-Mize E, McGiff JC. Renal cytochrome P450-related arachidonate metabolite inhibits (Na⁺ + K⁺)ATPase. *Nature*. 1985; 314(6012):620–2. [PubMed: 2986008]
16. Vio CP, Cespedes C, Gallardo P, Masferrer JL. Renal identification of cyclooxygenase-2 in a subset of thick ascending limb cells. *Hypertension*. 1997; 30(3 Pt 2):687–92. [PubMed: 9323006]
17. Vio CP, An SJ, Cespedes C, McGiff JC, Ferreri NR. Induction of cyclooxygenase-2 in thick ascending limb cells by adrenalectomy. *J Am Soc Nephrol*. 2001; 12(4):649–58. [PubMed: 11274225]
18. Cheng HF, Wang JL, Zhang MZ, Miyazaki Y, Ichikawa I, McKanna JA, et al. Angiotensin II attenuates renal cortical cyclooxygenase-2 expression. *J Clin Invest*. 1999; 103(7):953–61. [PubMed: 10194467]
19. Yang T, Singh I, Pham H, Sun D, Smart A, Schnermann JB, et al. Regulation of cyclooxygenase expression in the kidney by dietary salt intake. *Am J Physiol*. 1998; 274(3 Pt 2):F481–9. [PubMed: 9530264]
20. Nasrallah R, Landry A, Singh S, Sklepowicz M, Hebert RL. Increased expression of cyclooxygenase-1 and -2 in the diabetic rat renal medulla. *Am J Physiol Renal Physiol*. 2003; 285(6):F1068–77. [PubMed: 12888618]
21. Mangat H, Peterson LN, Burns KD. Hypercalcemia stimulates expression of intrarenal phospholipase A2 and prostaglandin H synthase-2 in rats. Role of angiotensin II AT1 receptors. *J Clin Invest*. 1997; 100(8):1941–50. [PubMed: 9329957]
22. Peterson LN, McKay AJ, Borzecki JS. Endogenous prostaglandin E₂ mediates inhibition of rat thick ascending limb Cl reabsorption in chronic hypercalcemia. *J Clin Invest*. 1993; 91(6):2399–407. [PubMed: 8390479]
23. Sands JM, Flores FX, Kato A, Baum MA, Brown EM, Ward DT, et al. Vasopressin-elicited water and urea permeabilities are altered in IMCD in hypercalcemic rats. *Am J Physiol*. 1998; 274(5 Pt 2):F978–85. [PubMed: 9612337]
24. Shiraishi N, Kitamura K, Kohda Y, Narikiyo T, Adachi M, Miyoshi T, et al. Increased endothelin-1 expression in the kidney in hypercalcemic rats. *Kidney Int*. 2003; 63(3):845–52. [PubMed: 12631065]
25. Peterson LN. Vitamin D-induced chronic hypercalcemia inhibits thick ascending limb NaCl reabsorption in vivo. *Am J Physiol*. 1990; 259(1 Pt 2):F122–9. [PubMed: 2115738]
26. Kos CH, Karaplis AC, Peng JB, Hediger MA, Goltzman D, Mohammad KS, et al. The calcium-sensing receptor is required for normal calcium homeostasis independent of parathyroid hormone. *J Clin Invest*. 2003; 111(7):1021–8. [PubMed: 12671051]
27. Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM, et al. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. *J Clin Invest*. 1997; 99(6):1399–405. [PubMed: 9077550]
28. Baud L, Oudinet JP, Bens M, Noe L, Peraldi MN, Rondeau E, et al. Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. *Kidney Int*. 1989; 35(5):1111–8. [PubMed: 2549293]

29. Neale TJ, Ruger BM, Macaulay H, Dunbar PR, Hasan Q, Bourke A, et al. Tumor necrosis factor- α is expressed by glomerular visceral epithelial cells in human membranous nephropathy. *Am J Pathol.* 1995; 146(6):1444–54. [PubMed: 7778683]
30. Jevnikar AM, Brennan DC, Singer GG, Heng JE, Maslinski W, Wuthrich RP, et al. Stimulated kidney tubular epithelial cells express membrane associated and secreted TNF α . *Kidney Int.* 1991; 40(2):203–11. [PubMed: 1942768]
31. Macica CM, Escalante BA, Connors MS, Ferreri NR. TNF production by the medullary thick ascending limb of Henle's loop. *Kidney Int.* 1994; 46(1):113–21. [PubMed: 7933828]
32. Kaji DM, Chase HS Jr, Eng JP, Diaz J. Prostaglandin E_2 inhibits Na-K-2Cl cotransport in medullary thick ascending limb cells. *Am J Physiol.* 1996; 271(1 Pt 1):C354–61. [PubMed: 8760065]
33. Stokes JB. Effect of prostaglandin E_2 on chloride transport across the rabbit thick ascending limb of Henle. Selective inhibitions of the medullary portion. *J Clin Invest.* 1979; 64(2):495–502. [PubMed: 457864]
34. Hebert SC, Brown EM, Harris HW. Role of the Ca^{2+} -sensing receptor in divalent mineral ion homeostasis. *J Exp Biol.* 1997; 200(Pt 2):295–302. [PubMed: 9050237]
35. Serros ER, Kirschenbaum MA. Prostaglandin-dependent polyuria in hypercalcemia. *Am J Physiol.* 1981; 241(3):F224–30. [PubMed: 6895150]
36. Hebert SC. Calcium and salinity sensing by the thick ascending limb: a journey from mammals to fish and back again. *Kidney Int Suppl.* 2004; (91):S28–33. [PubMed: 15461699]

Highlights

The effect of TNF on COX-2 expression was determined in the renal OM.

An elevation in urinary TNF was seen in WT mice given a vitamin D analog (DHT).

An increase in urinary PGE₂ was observed in WT mice treated with DHT.

COX-2 expression in the OM was elevated in WT but not TNF^{-/-} mice given DHT.

We demonstrated that during hypercalcemia COX-2 expression in the renal OM is TNF-dependent.

Figure 1A

Figure 1B

Fig 1. Food intake and body weight in WT and TNF^{-/-} mice ingesting DHT
 WT and TNF^{-/-} mice were maintained in metabolic cages and fed control diet for 4 days before being placed on DHT-containing diet for 7 days. **A.** Food consumption was monitored on a daily basis. The data represent means $\pm$ SEM, * p<0.05, ** p<0.01, *** p<0.001 vs. control (day 0) vs treatment day. **B.** Body weight was monitored on a daily basis. The data are shown as mean $\pm$ SEM. ** p<0.01, *** p<0.001 vs. control (day 0) vs treatment day.

Figure 2A

Figure 2B

Fig 2. DHT increases total serum Ca^{2+} levels and urinary Ca^{2+} excretion in WT and $\text{TNF}^{-/-}$ mice

WT and $\text{TNF}^{-/-}$ mice were maintained in metabolic cages and fed the control diet for 4 days before being placed on the DHT diet for 7 days. **A.** Serum Ca^{2+} levels, and **B.** urinary Ca^{2+} excretion was measured in mice fed control or DHT-containing diet for 7 days. The data are shown as mean $\pm$ SEM; n=3–6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ control (day 0) vs treatment day.

Fig 3. DHT increases urine volume in WT and TNF^{-/-} mice

WT and TNF^{-/-} mice were maintained in metabolic cages, fed control diet for 4 days and then placed on DHT diet for 7 days. Urine volume per cage was measured on a daily basis. The data are shown as mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ control (day 0) vs treatment day.

Fig 4. Water intake increased with DHT treatment

WT and TNF^{-/-} mice were maintained in metabolic cages, fed control diet for 4 days and placed on DHT containing diet for a period of 7 days, food and water intake were monitored on a daily basis. The data represent mean $\pm$ SEM. * p<0.05, ** p<0.01, *** p<0.001 control (day 0) vs treatment day.

Fig 5. DHT increases urinary TNF levels

WT mice were maintained in metabolic cages, fed control diet for 4 days, and then placed on DHT-containing diet for 7 days; urine was collected daily and analyzed for TNF levels. The data are shown as mean $\pm$ SEM; n=9 cages. * $p<0.05$, ** $p<0.01$ control (day 0) vs treatment day.

Fig 6. COX-2 expression in outer medulla of WT mice is elevated in response to DHT treatment
WT mice were fed control diet for 4 days then given a DHT-containing diet for 1, 3, or 7 days. Expression of COX-2 in the OM was analyzed by Western blot. The top panel shows a representative Western blot and the bottom panel shows densitometry analysis after COX-2 expression was normalized to β-actin levels. The data are shown as mean ± SEM, n=4. * p<0.05 control (day 0) vs treatment day.

Fig 7. DHT increases COX-2 expression in WT but not $TNF^{-/-}$ mice

WT mice (A) and $TNF^{-/-}$ mice (B) were fed control or DHT-containing diet for 7 days. Expression of COX-2 in the OM was analyzed by Western blot. The top panels are representative Western blots and the bottom panels show densitometry analysis of COX-2 expression normalized to β -actin levels. The data are shown as mean $\pm$ SEM, n=5. * $p < 0.05$.

Fig 8. Effects of DHT on urinary PGE₂ levels in WT and TNF^{-/-} mice

A) WT mice (n=9 cages) and **B)** TNF^{-/-} mice (n=3 cages) were maintained in metabolic cages, fed control diet for 4 days, and then placed on DHT-containing diet for 7 days. Urine was collected daily and analyzed for PGE₂ levels. The data are shown as mean $\pm$ SEM; * p<0.05, ** p<0.01 control (day 0) vs treatment day.