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Eicosanoids and Tumor Necrosis Factor-alpha in the kidney

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Abstract

The thick ascending limb of Henle's loop (TAL) is capable of metabolizing arachidonic acid (AA) by cytochrome P450 (CYP450) and cyclooxygenase (COX) pathways and has been identified as a nephron segment that contributes to salt-sensitive hypertension. Previous studies demonstrated a prominent role for CYP450-dependent metabolism of AA to products that inhibited ion transport pathways in the TAL. However, COX-2 is constitutively expressed along all segments of the TAL and is increased in response to diverse stimuli. The ability of Tamm-Horsfall glycoprotein, a selective marker of cortical TAL (cTAL) and medullary (mTAL), to bind TNF and localize it to this nephron segment prompted studies to determine the capacity of mTAL cells to produce TNF and determine its effects on mTAL function. The colocalization of calcium-sensing receptor (CaR) and COX-2 in the TAL supports the notion that activation of CaR induces TNF-dependent COX-2 expression and PGE₂ synthesis in mTAL cells. Additional studies showed that TNF produced by mTAL cells inhibits ⁸⁶Rb uptake, an *in vitro* correlate of natriuresis, in an autocrine- and COX-2-dependent manner. The molecular mechanism for these effects likely includes inhibition of Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2) expression and trafficking.

Keywords

Eicosanoids; COX-2; calcium-sensing receptor; TNF; NKCC2; NFAT5

I) COX-2 expression in the thick ascending limb of Henle's loop

The thick ascending limb of Henle's loop (TAL) is water impermeable and reabsorbs approximately 25% of filtered NaCl. These features facilitate the maintenance of medullary interstitial osmolality, essential for conservation of the renal countercurrent exchange system and the ability of the kidney to concentrate urine. The transport of NaCl across the apical membrane of the TAL occurs mainly via a bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter, NKCC2, which is exclusively expressed along the TAL and is the molecular target of loop

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diuretics¹⁻⁵. The reabsorption of NaCl is then completed as Na⁺ exits the cell via the basolateral Na⁺-K⁺-ATPase while Cl⁻ diffuses along its electrochemical gradient through basolateral KCl cotransporters or Cl⁻ channels (Figure 1). Accordingly, the TAL is a nephron site where variations in Na⁺ reabsorption may influence long-term regulation of blood pressure and susceptibility to cardiovascular disease⁶. For instance, increased reabsorption in this nephron segment contributes to salt-sensitive hypertension⁷. Conversely, mutations of the NKCC2 gene (*SLC12A1*) demonstrated to cause reduced NKCC2 activity, and detected by screening more than 3000 members of the Framingham Heart Study, were associated with a significant reduction in blood pressure and risk of death due to cardiovascular disease⁸; several of these mutants exhibited impaired processing and altered transport characteristics⁹. Moreover, patients with inactivating mutations of NKCC2 exhibit a severe salt and water wasting phenotype (Bartter syndrome), demonstrating the role played by this transporter in the regulation of extracellular fluid volume homeostasis¹⁰. Thus, a small but chronic reduction in NKCC2 function minimizes cardiovascular risk and is associated with lower blood pressure in otherwise healthy individuals. As subtle alterations in sodium homeostasis may contribute to the development of hypertension, it is important to identify regulatory systems affect TAL cell function, as reabsorptive capacity is limited downstream of this segment and inhibition of NKCC2 results in marked natriuresis and diuresis.

The TAL is capable of metabolizing arachidonic acid (AA) by cytochrome P450 (CYP450) and cyclooxygenase (COX) pathways. Our initial studies demonstrated a prominent role for CYP450-dependent metabolism of AA to products that inhibited ion transport pathways in the TAL¹¹⁻¹⁴. However, detection of a small percentage of TAL cells, including cortical TAL (cTAL), macula densa, and medullary TAL (mTAL), expressing COX-2 in normal kidney suggested that regulation of TAL cell function may not be not limited to effects of CYP450-derived metabolites¹⁵⁻¹⁷. In addition to constitutive expression, *in vivo* COX-2 expression along the TAL increases during treatment with angiotensin converting enzyme inhibitors, neonatal development, adrenalectomy, changes in salt intake, and diabetes^{15, 18-25}. Interestingly, COX-2 expression increases axially along the TAL following adrenalectomy and is associated with increased PGE₂ synthesis²². Morphometric analysis demonstrated that 1.6 ± 0.3% of TAL cells were stained for COX-2 in sham-operated rats whereas after adrenalectomy 30.6 ± 4.7% of TAL cells expressed COX-2. Expression of enzymatically active COX-2 was observed in 1.4 ± 0.2% of TAL cells after dexamethasone was given to adrenalectomized rats²². The tubules were identified as TAL segments based on the colocalization of COX-2 and the Tamm-Horsfall glycoprotein (THP), which is a specific marker of cTAL and mTAL cells, using single immunolabeling in serial consecutive sections and double immunolabeling in the same tissue sections. Also of note is the nearly continuous staining of TAL epithelial cells intermingled with a few COX-2 negative TAL cells after adrenalectomy, or during postnatal renal development²⁰, an expression pattern reminiscent of the recruitment phenomena observed for renin-containing myoepithelial cells in afferent arterioles²⁶. Localization of COX-2 along the nephron also can be defined by the anatomical relationship of vascular and renal structures in the kidney. For instance, the arcuate artery defines the boundaries between the cortex and medulla, and thus, the correlation between the localization of an arcuate artery and juxtamedullary nephrons provided evidence for cTAL and mTAL that were heavily stained for COX-2 compared to unstained neighboring tubular cells¹⁷. Importantly, studies regarding the regulation of COX-2 expression by inhibition of the renin-angiotensin system in neonates clearly demonstrate localization of COX-2 to the mTAL as well as the cTAL²⁷. Expression of COX-2 in the mTAL also was evident in models of sepsis and diabetes^{25, 28}. Using a cecal ligation and puncture model of sepsis, a precise analysis of COX-2 expression along the TAL and other renal structures was provided by confocal microscopy and fluorescence-based assessments to identify renal structures. This elegant study clearly revealed that Toll-

like receptor 4-dependent increases in COX-2 expression were restricted mostly to THP-expressing cTAL and mTAL segments, and is similar to another study in which administration of *Escherichia coli* lipopolysaccharide (LPS) increased COX-2 in the renal cortex, outer medulla, and inner medulla^{28, 29}. Expression of COX-2 increased in the mTAL, outer medullary collecting duct, and inner medullary collecting duct 4 and 6 wk after streptozotocin injection, an effect that was reversed after treatment with insulin and appears to involve a post-transcriptional mechanism²⁵. Collectively, these data demonstrate that the entire length of the TAL has the capacity to express COX-2 and are consistent with earlier studies demonstrating COX-2 expression in primary cultures of mTAL cells in response to several stimuli^{17, 30–32}. Thus, COX-2-derived eicosanoids may contribute to functions critical to the TAL segment of the nephron³³.

II) Functional implications of COX-2 regulation by calcium-sensing receptor (CaR) and TNF in the mTAL

Hypertension is a major independent risk factor for heart attack, stroke, and end-stage renal disease. Complex genetic and environmental factors contribute to the pathogenesis of essential hypertension, and studies have demonstrated a positive correlation between salt intake and elevated blood pressure^{34, 35}. Moreover, long-term use of COX-2 inhibitors such as rofecoxib and celecoxib were associated with hypertension and increased thrombotic events including myocardial infarction and stroke³⁶. The kidney plays an important role in the development and maintenance of hypertension and mutations of various transporter molecules have been linked to hypertension³⁷. However, these mutant proteins explain mostly rare forms of hypertension, which account for 1–2% of all cases. Several molecules regulate transport function in the TAL by autocrine and paracrine pathways³⁸. Clearly, additional mechanisms involving locally produced molecules that can regulate transport function remain to be uncovered. Recently, a locus in the 5' region of uromodulin (Tamm-Horsfall protein), which is exclusively expressed in the TAL and binds TNF, was associated with a lower risk of hypertension suggesting a putative role of this variant in hypertension via regulation of sodium balance^{39–41}.

The kidney exhibits high levels of TNF mRNA in response to LPS challenge⁴², and our previous work showed that TNF produced by mTAL cells inhibits ⁸⁶Rb uptake, an *in vitro* correlate of natriuresis, in an autocrine- and COX-2-dependent manner^{43–45}. However, the notion that TNF exerts regulatory functions in this region of the kidney is understudied. Indeed, the effects of TNF in the kidney are complex and mirror the duality of this cytokine in the immune system as a mediator of innate immunity and inflammation. For instance, TNF is a proinflammatory cytokine that contributes to septic shock and chronic inflammatory diseases. However, local TNF production subserves critical host defense mechanisms by increasing vascular permeability and facilitating blood clotting in small blood vessels, which prevents pathogens from entering the bloodstream. Accordingly, while neutralizing excess TNF is an effective therapy for Crohn's disease and rheumatoid arthritis, treatment with anti-TNF reagents leaves *M. tuberculosis*-infected individuals at increased risk for reactivation of latent tuberculosis infection^{46, 47}. Moreover, loss of physiological TNF production is associated with glomerulonephritis in autoimmune-prone NZB mice⁴⁸. Accordingly, we propose that the paradigm of beneficial versus detrimental effects of TNF also is evident in the cardiovascular and renal systems. Several studies indicate that inhibition of TNF has a beneficial effect in models of malignant hypertension associated with inflammation. For instance, the TNF antagonist, etanercept, reduced Ang II-dependent increases in blood pressure in C57BL/6J mice, lowered mean arterial pressure in a rat model of pregnancy-induced hypertension, prevented the development of hypertension in fructose-fed rats, and decreased blood pressure in a murine model of lupus^{49–52}. In some instances however, the beneficial effects on renal inflammation and damage occurred independently

of an effect on blood pressure, as in double transgenic rats expressing human renin and angiotensinogen where etanercept reduced mortality, albuminuria, and renal fibronectin expression without affecting systolic blood pressure⁵³. Similarly, etanercept had a transient inhibitory effect on blood pressure, observed on days 6–11 after infusion of Ang II while blood pressure was similar in control and etanercept-treated groups on days 1–5 and 12–15⁵⁴. Interestingly, etanercept reduced renal damage and inflammation assessed on day 15 despite the lack of a difference in blood pressure. Etanercept also failed to decrease blood pressure in the deoxycorticosterone acetate (DOCA)-salt model of hypertension, although it was associated with a reduction in albuminuria, and attenuated urinary excretion of MCP-1 and endothelin-1, suggesting that TNF contributes to renal inflammation associated with mineralocorticoid-induced hypertension⁵⁵. Moreover, TNF increases renal vascular resistance and arterial pressure during pregnancy, but does not increase blood pressure in non-pregnant female or normal male rats, suggesting that TNF increases blood pressure in a context-dependent manner^{56,57}. In addition to these functions of TNF, our laboratory has studied the local production of TNF by mTAL cells and described a mechanism involving COX-2 that inhibits Na⁺ transport in these cells. Exogenous TNF, as well as TNF formed after challenge of mTAL cells with LPS, inhibited ⁸⁶Rb uptake via a prostaglandin-dependent mechanism^{43,44}. The effects of TNF on ion transport was related to induction of COX-2-dependent PGE₂ synthesis³⁰. These findings suggest that TNF acts in an autocrine manner to inhibit ion transport in the mTAL via a COX-2-dependent mechanism that increases PGE₂ synthesis. Recent *in vivo* studies have shown that TNF is natriuretic as systemic administration of human recombinant TNF to mice increased absolute and fractional excretion of sodium^{58,59}. This renal tubular effect, possibly mediated by activation of TNF receptor 1 (TNFR1), is consistent with the presence of TNFR1 mRNA in renal outer medulla and cortex⁶⁰. Moreover, the failure of TNFR1^{-/-} mice to show an appropriate increase in FE_{Na}% may be a consequence of reduced renal tubular effects of TNF in mice with genetic deletion of TNFR1⁶⁰. This was confirmed by the observation that TNFR1^{-/-} mice excreted less urinary sodium in response to Ang II infusion compared with WT mice.

Consideration of the mechanisms that regulate TNF production in various cell types provided important clues for identifying candidate molecules that might contribute to production of this cytokine and increase COX-2 expression in the TAL. For instance, TNF gene transcription involves multiple cellular-specific signaling factors including an increase in intracellular calcium concentrations (Ca²⁺) and protein kinase C (PKC) activation^{61–63}. Since activation of calcium-sensing receptor (CaR) increases Ca²⁺ and PKC activity^{64,65}, and inhibits apical K⁺ channel activity in the TAL via a CYP450-dependent mechanism^{66,67}, we hypothesized that TNF production in mTAL cells also may be increased following CaR activation. We demonstrated that mTAL cells in primary culture expressed CaR and produced TNF following its activation^{31,68–70}. Moreover, immunohistochemical analysis revealed that CaR and COX-2 are colocalized in TAL tubules. Note that CaR is present on basolateral membranes of TAL cells, and each cell expressing COX-2 co-expresses CaR (Figure 2). These data are consistent with our studies showing that activation of CaR in mTAL cells increases COX-2 expression and PGE₂ synthesis and inhibits ⁸⁶Rb uptake in a TNF- and COX-2-dependent manner^{31,68}. The precise contribution of COX-2 pathways to TAL cell functions such as urine concentration and dilution upon activation of the CaR remains to be determined. Interestingly, genetic deletion of TNF is associated with higher ambient urine osmolality and greater diluting ability of the kidney, which is consistent with effects of CaR activation on urinary concentrating ability^{64,71}.

In addition to effects on water homeostasis, we propose that TNF and COX-2-derived PGE₂ synthesis may contribute to a mechanism that regulates NaCl transport in the TAL^{31,68,72}.

This hypothesis is supported by increased CaR activity in Bartter syndrome, which is characterized by a severe reduction in salt reabsorption by the TAL¹⁰. In particular, Type V Bartter syndrome is caused by gain-of-function mutations of the CaR, and treatment with COX inhibitors can reduce urine volume and excretion of calcium in antenatal Bartter syndrome (also called hyperprostaglandin E syndrome), characterized by severe polyuria and dramatically elevated levels of PGE₂ in the blood and urine^{73–76}. Moreover, COX-2 inhibition causes Na⁺ retention in some human subjects and decreases urinary Na⁺ excretion in subjects with salt depletion or in elderly subjects on a normal Na⁺ diet^{77–82}. These and other findings are consistent with observations that, under certain conditions, COX-2 inhibitors elevate blood pressure^{78, 83–85}. Salt loading in experimental models increases COX-2 expression in the medulla and the subsequent increase in PGE₂ contributes to a natriuretic mechanism that is antagonized by COX-2 inhibitors^{24, 86}. As the functional importance of PGE₂ to the mTAL, a nephron segment crucial to regulation of salt and water balance, has been demonstrated by several investigators^{30, 87–90}, increased COX-2 expression and PGE₂ production in the mTAL in response to CaR activation and subsequent to production of TNF also may contribute to regulate salt and water homeostasis in the TAL (Figure 3).

III) NKCC2 as a molecular target for TNF-dependent COX-2-derived PGE₂

Understanding the mechanisms underlying the regulation of NKCC2 is essential as subtle differences in the regulation of renal transport proteins may influence blood pressure homeostasis. Both CYP450- and COX-2-derived eicosanoids produced by the mTAL affect NKCC2 function^{14, 89}. For instance, subnanomolar concentrations of PGE₂ inhibit NKCC2 activity in mouse mTAL cells by a mechanism involving a decrease in the number of functioning cotransporter molecules⁸⁹. However, the molecular mechanisms that account for the inhibitory effect of PGE₂ are not well defined. As detailed in a recent review article describing the relationship between NKCC2 and the CaR in the TAL, activation of CaR directly affects NaCl reabsorption via NKCC2⁹¹. Moreover, activation of CaR has been described to exhibit characteristics of a furosemide-like effect⁹². Our recent studies showed that inhibition of apical chloride uptake in response to CaR activation in primary cultured mTAL cells is nuclear factor of activated T cells (NFAT) 5-dependent; NFAT5 is also known as tonicity-responsive enhancer/osmotic-response element-binding protein (TonEBP/OREBP), a transcription factor crucial for cellular responses to hypertonic stress^{93–96}. As NFAT5 activation increases TNF production in mTAL cells, and since chloride uptake in this nephron segment occurs mainly via NKCC2, we tested the hypothesis that TNF acts as an endogenous inhibitor of NKCC2 *in vivo*. Western blot analysis indicated there was approximately a two-fold increase in NKCC2 expression in the outer medulla obtained from TNF^{-/-} mice compared with WT mice⁷¹. Although a single gene encodes the NKCC2 protein, differential splicing of NKCC2 pre-mRNA results in formation of three major isoforms, NKCC2A, NKCC2B, and NKCC2F, arising from variable exon 4^{97, 98}. A prominent difference between the isoforms is their relative affinities for chloride; namely, high affinity NKCC2B and NKCC2A versus low affinity NKCC2F. The increasing isoform affinities and localization correlates with the luminal concentrations of Na⁺, K⁺, and Cl⁻ along the TAL, a function of progressively dilute urine in the tubule lumen proceeding from medulla to cortex. Although a role for the NKCC2B isoform in the regulation of renin has been demonstrated, relatively little is known about the physiological responses attributed to NKCC2 isoforms^{99, 100}. Analysis by qRT-PCR revealed an approximate four-fold increase in NKCC2A mRNA accumulation in the outer medulla from TNF^{-/-} compared with WT mice that was markedly attenuated when TNF^{-/-} mice were treated with hTNF⁷¹. Neither NKCC2F mRNA accumulation nor NKCC2B mRNA accumulation was affected by TNF gene deletion or treatment with hTNF, suggesting that compensatory changes in these isoforms are not evident in TNF^{-/-} mice⁷¹. Bumetanide-sensitive oxygen consumption, an

in vitro correlate of NKCC2 activity also was elevated in mTAL tubules from TNF^{-/-} compared with WT mice, an effect that was negated by treatment of TNF^{-/-} mice with hTNF. The finding that ambient urinary osmolality is higher in TNF^{-/-} compared with WT mice is consistent with an essential role of NKCC2 in establishing the medullary interstitial gradient in studies using NKCC2^{-/-} mice, which exhibit a lower ambient urinary osmolality than their WT littermates¹⁰¹. The TAL is the major segment where the separation of water and solute occurs and, therefore, also is involved in the diluting ability of the kidney. Accordingly, urine osmolality, determined before and 1h after WT and TNF^{-/-} mice were water loaded by oral gavage, was greater in TNF^{-/-} compared with WT mice. The cTAL has a higher capacity for urine dilution compared with the mTAL^{102, 103}. This coincides with the presence of NKCC2A and NKCC2B isoforms in the cTAL, as previously described^{2, 100} and supports the notion that elevation in NKCC2A in TNF^{-/-} mice contributes to the greater diluting capacity observed in these mice⁷¹. Therefore, TNF may be part of a repressor mechanism in the TAL that limits NKCC2A mRNA accumulation by regulating alternative splicing or exerting a post-transcriptional effect on mRNA half-life. Since TNF is a regulator of COX-2 expression in the mTAL, the impact of defective COX-2-derived PGE₂ synthesis to the regulation of NKCC2 in TNF deficient mice remains to be determined (Figure 3).

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Figure 1. Major transport pathways in the TAL

Selected transporter molecules in the TAL that contribute to NaCl reabsorption in this segment of the nephron include the apically expressed NKCC2 and ROMK K^+ channel; the latter is important for K^+ recycling that helps maintain NKCC2 function. Also note the basolateral localization of the CaR, $\text{Na}^+-\text{K}^+-\text{ATPase}$, and CLC-K chloride channel. The dashed arrow indicates paracellular transport of ions.

Figure 2. Colocalization of Calcium-sensing Receptor and COX-2 in the TAL

Double label immunostaining of TAL segments for COX-2 (blue) and Calcium-sensing Receptor (CaR) (brown); a similar protocol was used in our previous study²². CaR is observed on the basolateral membrane, and each cell expressing COX-2 co-expresses CaR; scale bars = 50 μ m. Tissue sections were observed and photographed on a Nikon Eclipse 600 microscope with a Nikon DXM1200 digital photographic system (Nikon Corporation, Tokyo, Japan).

Figure 3. Schematic of the CaR/TNF/COX-2 regulatory system in the TAL

The diagram shows that activation of CaR increases TNF production, via activation of the NFAT5 (Ton/EBP) transcription factor, which subsequently increases COX-2-derived PGE₂ synthesis. The regulation of NKCC2 by TNF is postulated to include a mechanism involving COX-2 as well as a COX-independent pathway that may be important under basal conditions.