Calcium-sensing receptor-mediated TNF production in medullary thick ascending limb cells

DAIRONG WANG, PAULINA L. PEDRAZA, HUDA ISMAIL ABDULLAH, JOHN C. MCGIFF, AND NICHOLAS R. FERRERI
Department of Pharmacology, New York Medical College, Valhalla, New York 10595

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Calcium-sensing receptor-mediated TNF production in medullary thick ascending limb cells. Am J Physiol Renal Physiol 283: F963–F970, 2002. First published July 16, 2002; 10.1152/ajprenal.00108.2002.—Medullary thick ascending limb (mTAL) cells in primary culture express the Ca2+-sensing receptor (CaR), a G protein-coupled receptor that senses changes in extracellular Ca2+ (Ca2o) concentration, resulting in increases of intracellular Ca2+ concentration and PKC activity. Exposure of mTAL cells to either Ca2o or the CaR-selective agonist poly-L-arginine increased TNF-α synthesis. Moreover, the response to Ca2o was enhanced in mTAL cells transfected with a CaR overexpression vector. Transfection of mTAL cells with a TNF promoter construct revealed an increase in reporter gene activity after exposure of the cells to Ca2o, suggesting that intracellular signaling pathways initiated by means of activation of a CaR contribute to TNF synthesis by a mechanism that involves transcription of the TNF gene. Neutralization of TNF activity with an anti-TNF antibody attenuated Ca2o-mediated increases in cyclooxygenase-2 (COX-2) protein expression and PGE2 synthesis, suggesting that TNF exerts an autocrine effect in the mTAL, which contributes to COX-2-mediated PGE2 production. Preincubation with the PKC inhibitor bisindolylmaleimide I inhibited Ca2o-mediated TNF production. Significant inhibition of COX-2 protein expression and PGE2 synthesis also was observed when cells were challenged with Ca2o in the presence of bisindolylmaleimide I. The data suggest that increases in TNF production subsequent to activation of the CaR may be the basis of an important renal mechanism that regulates salt and water excretion.

IN PREVIOUS STUDIES, WE HAVE identified two pathways of arachidonic acid metabolism in the rat medullary thick ascending limb (mTAL) that regulate ion transport in this nephron segment. Each pathway was activated by either ANG II or Ca2o and showed a short-term response (15 min) in which 20-HETE acted and a long-term response (>2–3 h) in which PGE2 was the principal product (9, 34). Furthermore, mTAL cells have been shown to synthesize TNF-α and respond to exogenous TNF by expressing cyclooxygenase-2 (COX-2) (8, 20). After a several-hour latent period, PMA, a PKC activator, also induced expression of COX-2 in mTAL cells, suggesting a PKC link to the response. We concluded that short-term regulation of ion transport in the mTAL occurred in a COX-2-independent manner, whereas long-term regulation was TNF and COX-2 dependent. These findings dictated the experimental design of the present study, namely, to determine the role of TNF production by the mTAL in mediating COX-2 expression in response to stimulation of the Ca2+-sensing receptor (CaR) and whether PKC contributes to production of TNF by the mTAL.

The mTAL of Henle’s loop is the site of action for loop diuretics and reabsorbs −25% of filtered NaCl. It also is responsible for the generation of concentrated or dilute urine during antidiuresis and water diuresis, respectively. Na+, K+, and Cl− are reabsorbed from the tubular fluid via the Na+/K+/2Cl− cotransporter on the apical membrane while K+ is recycled back to the tubular fluid via apical K+ channels. mTAL cells produce TNF after challenge with LPS or ANG II (9, 20). This cytokine, which increases COX-2-mediated PGE2 production, contributes to a cytokine- and COX-2-dependent mechanism that inhibits 86Rb uptake in mTAL cells, an in vitro correlate of natriuresis (8). TNF gene transcription involves multiple cellular-specific signaling factors, including an increase in intracellular Ca2+ concentrations and PKC activation (12, 14, 18).

CaRs are expressed in tissues, including the kidney, that are involved in Ca2+ homeostasis (26, 27). CaRs are G protein-coupled receptors that transduce Ca2+ binding into several intracellular signals, including stimulation of inositol triphosphate (IP3) production and DAG levels. IP3 facilitates Ca2+ release from intracellular stores, and DAG increases PKC activity (3). Activation of the CaR inhibits K+ recycling in the mTAL by a 20-HETE-dependent mechanism (34). Our laboratory recently showed that activation of the CaR in primary cultures of mTAL cells increased PGE2 synthesis by means of a COX-2-dependent mechanism (31). In the present study, we demonstrate that the increase in COX-2 expression and PGE2 synthesis is differentially dependent on increased TNF production.
subsequent to activation of the CaR. The increases in TNF production as well as COX-2 protein expression and PGE₂ production are PKC dependent.

**METHODS**

**Animals.** Male Sprague-Dawley rats (100–110 g; Charles River Lab, Wilmington, MA) were maintained on standard rat chow (Ralston-Purina, Chicago, IL) and given tap water ad libitum.

**Reagents.** Tissue culture media was obtained from Life Technologies (Grand Island, NY). Reagent-grade chemicals and collagenase (type 1A) were from Sigma (St. Louis, MO). Polyvinylidene difluoride membranes were obtained from Amersham (Arlington Heights, IL). Reagents for preparation of the TNF-a ELISA kit were purchased from Pharmingen (San Diego, CA). PGE₂ ELISA kits were from Neogen (Lexington, KY). The neutralizing anti-TNF antibody was purchased from R&D Systems (Minneapolis, MN). The β-galactosidase enzyme assay system and luciferase assay kit were from Promega (Madison, WI). The pGV-B2-TNFprom promoter construct was a generous gift from Dr. Akio Nakamura (Tokyo University, School of Medicine, Tokyo, Japan). The isolation and characterization of mTAL cells. The isolation and characterization of mTAL cells (~95% purity) were performed as previously described (6, 20). Briefly, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.65 mg/100 g body wt). The kidneys were perfused with sterile 0.9% saline via retrograde perfusion of the aorta and cut along the corticopapillary axis. The inner stripe of the outer medulla was excised, minced with a sterile blade, and incubated for 10 min at 37 °C in a 0.1% collagenase solution gassed with 95% oxygen. The suspension was filtered through a 52-µm nylon mesh membrane (Fisher Scientific, Springfield, NJ). The filtered solution was discarded, and the tubules retained on the mesh were resuspended in HBSS. Then, the solution was centrifuged at 500 rpm for 5 min, the supernatant was aspirated, and the cells were cultured in 1:1 DMEM/Ham’s F-12 medium (GIBCO-BRL), 10% FBS (Gibco), 20 ng/ml epidermal growth factor (Life Technologies), 100 U/ml streptomycin-penicillin (GIBCO-BRL), 2 mM L-glutamine, gentamycin sulfate, and 1 µg/ml fungizone (Gibco). After 3 days, monolayers of cells were 80–90% confluent. The cells were quiesced for 24 h in RPMI containing 0.42 mM CaCl₂ and 0.5% FBS, 2 mM L-glutamine, 100 U/ml streptomycin-penicillin, MEM nonessential amino acids (GIBCO-BRL), MEM sodium pyruvate, and 2-mercaptoethanol before their use. In all experiments, control conditions (i.e., no addition of CaCl₂) reflect that cells were incubated in medium containing 0.42 mM Ca²⁺. This amount of Ca²⁺ should be added to the amounts used to challenge the cells to obtain total Ca²⁺ concentrations in the media during the transfection period. The membranes were placed in blocking solution containing 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature (RT) for 30 min. Membranes were immunoblotted with either a rabbit anti-mouse COX-2 polyclonal antibody (Cayman) or monoclonal anti-β-actin (clone AC-15; Sigma) for 1 h at RT. The membranes were then washed three times with Tris-buffered saline containing 0.1% Tween 20 and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 30 min at RT. Membranes were washed, and enhanced chemiluminescence was used to evaluate protein expression. The blots were then scanned, and relative intensities were determined with Image software (National Institutes of Health), which was calibrated by using an internal standard (Kodak). The expression of β-actin was used to correct for variation in sample loading.

**Measurement of TNF.** Primary cultured rat mTAL cells were quiesced overnight in RPMI 1640 containing 0.5% FCS. Cells were challenged with CaCl₂ or poly-L-arginine for different times at 37°C and 5% CO₂, and TNF levels in supernatants were determined by ELISA (Pharmingen) according to the manufacturer’s protocol.

**Gene transfection.** mTAL cells were cultured to 70–80% confluence. The medium was removed, and cells were placed in 1 ml of serum-free OPTI-MEM medium containing 3 µg/well of either plasmid DNA containing the CaR (a kind gift from Dr. Karin Rodland, University of Oregon) or empty plasmid vector (pcDNA3.1) and 10 µl lipofectamine reagent (Life Technologies) for 4 h at 37°C and 5% CO₂. After the transfection period, 1 ml of DMEM/F-12 containing 20% FCS was added, and the cells were incubated overnight at 37°C and 5% CO₂. The medium was then removed, and cells were cultured for an additional 12 h in DMEM/F-12 containing 10% FCS. The cells were quiesced overnight in RPMI medium containing 0.5% FCS and then treated with the appropriate reagents for the indicated times; TNF levels in the supernatants were determined by ELISA.

**Analysis of Ca²⁺ effects on TNF gene regulation.** Transcriptional regulation of TNF production was assessed with a rat TNF promoter construct that drives expression of a luciferase reporter gene in the pGV-B2 plasmid (21). mTAL cells were cotransfected with 1 µg/ml of pGV-B2-TNFprom and 1 µg/ml pact-β-galactosidase in the presence of 10 µl lipofectamine reagent and 990 µl OPTI-MEM for 4 h at 37°C and 5% CO₂. Luciferase activity was normalized to account for differences in transfection efficiency by cotransfecting cells with pact-β-galactosidase-expressing plasmid. Data were expressed as the increase of luciferase activity/β-galactosidase activity vs. control.

**Statistical analysis.** The responses were compared by unpaired Student’s t-test or by a one-way ANOVA followed by the Newman-Kuels test when multiple comparisons were made. Data are presented as means ± SD; P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Effects of Ca²⁺ and the CaR agonist poly-L-arginine on mTAL TNF production.** Increases in PKC activity and intracellular levels of Ca²⁺ have been associated...
with an increase in TNF production (7, 13). As activation of the CaR increases PKC activity and intracellular Ca\(^{2+}\) levels (27), we determined the effects of CaR stimulation on TNF production by primary cultures of mTAL cells. Cells exhibited a significant increase in TNF production that was observed after exposure to 1.0 mM CaCl\(_2\) at each of the time points tested (Fig. 1A). Moreover, TNF production was maximal after addition of 1.0 mM CaCl\(_2\), except for the 3-h time period in which maximal production was observed after addition of 1.2 mM CaCl\(_2\). Release of TNF into the media was time dependent and was near maximal by ~6 h after addition of CaCl\(_2\). Challenge with poly-l-arginine, a CaR-selective agonist, for 9 h also increased TNF production by mTAL cells (Fig. 1B). These data indicate that TNF production by mTAL cells was increased on activation of the CaR.

Role of the CaR in TNF production in mTAL cells. The contribution of the CaR to TNF production was further assessed by overexpressing the receptor in mTAL cells and measuring the response to Ca\(^{2+}\). mTAL cells were transfected with plasmid DNA expressing the CaR (pcDNA3-CaR) or the corresponding empty plasmid vector without the CaR gene (pcDNA3). Cells were quiesced and treated with 1.2 mM CaCl\(_2\) for 9 h, and TNF production was determined. TNF production was similar in cells challenged with pcDNA3 or pcDNA3-CaR in the absence of CaCl\(_2\) (Fig. 2). However, TNF synthesis was ~50% greater in cells transfected with pcDNA3-CaR compared with cells transfected with pcDNA3 (empty vector) after challenge with 1.2 mM Ca\(^{2+}\) (Fig. 2). These data are consistent with the notion that the enhanced TNF production response to Ca\(^{2+}\) is mediated by means of activation of CaRs in mTAL cells.

Ca\(^{2+}\) increases activity of a TNF promoter construct. TNF gene transcription is inducible and regulated in a cell type-specific manner. The possible contribution of TNF gene transcription to the observed increases in TNF production in response to Ca\(^{2+}\) was evaluated in mTAL cells transfected with the TNF promoter construct pGV-B2-TNFprom. An increase in luciferase activity was observed after addition of either 1.0 or 1.2 mM Ca\(^{2+}\) for 9 h (Fig. 3). Addition of 1.0 mM Ca\(^{2+}\) increased luciferase activity by ~50% in cells transfected with pGV-B2-TNFprom (Fig. 3, inset). In contrast, no luciferase activity was observed when cells were transfected with a control plasmid lacking the TNF promoter construct (control pGV-B2) and then incubated in either the absence or presence of 1.2 mM Ca\(^{2+}\) (Fig. 3). Moreover, the β-galactosidase construct, which was used to normalize luciferase expression, had no endogenous luciferase activity (Fig. 3). These data suggest that transcriptional regulation of the TNF gene in mTAL cells was observed after activation of the CaR.

TNF contributes to Ca\(^{2+}\)-mediated increases in PGE\(_2\) production. Exogenous TNF induces transient increases in COX-2 mRNA accumulation and protein expression in mTAL cells (8). Thus the contribution of TNF, synthesized by mTAL cells, to increases in PGE\(_2\) Fig. 1. Characterization of Ca\(^{2+}\)-mediated TNF production. Primary cultures of medullary thick ascending limb (mTAL) cells were quiesced for 24 h in RPMI 1640 containing 0.42 mM CaCl\(_2\) and 0.5% FBS and then exposed for 3, 6, or 9 h to increasing concentrations of Ca\(^{2+}\) (A) or 100 nM poly-l-arginine (B). Supernatants were collected and TNF levels were determined by ELISA; data were normalized for protein content. Note that values on the x-axis reflect concentrations of CaCl\(_2\) added to cells that already contained 0.42 mM CaCl\(_2\) in the media. *P < 0.01, **P < 0.001; n = 4.
production after challenge with Ca\textsuperscript{2+} was evaluated by using a neutralizing concentration of anti-TNF antisera. Cells were exposed to Ca\textsuperscript{2+} in the absence or presence of the antisera. Addition of the anti-TNF antisera did not affect basal PGE\textsubscript{2} production but inhibited Ca\textsuperscript{2+}-mediated increases in PGE\textsubscript{2} production by ~35–40% (Fig. 4). In contrast, addition of purified IgG (isotype control) had no effect on PGE\textsubscript{2} production by cells incubated in the absence or presence of Ca\textsuperscript{2+} (Fig. 4). The possibility that TNF contributed to increased COX-2 protein expression in cells challenged with 1.2 mM CaCl\textsubscript{2} but had no effect on expression in unstimulated cells (Fig. 5). Normal rabbit serum was without effect (data not shown). These data suggest that an autocrine mechanism involving TNF contributed to Ca\textsuperscript{2+}-mediated increases in COX-2 expression and PGE\textsubscript{2} production, although COX-2 expression was suppressed more than PGE\textsubscript{2} synthesis after treatment with anti-TNF.

Fig. 2. Effects of CaR overexpression on TNF production. mTAL cells were transfected with 3 \(\mu\)g/well of the CaR plasmid (pcDNA3-CaR) or empty vector (pcDNA3) and then treated with 1.2 mM CaCl\textsubscript{2} for 9 h. TNF levels were measured by ELISA and then normalized by the protein amount in each sample (\(n = 3\)). An additional 0.42 mM CaCl\textsubscript{2} was present in the media for each condition tested.

Fig. 3. Effects of Ca\textsuperscript{2+} on luciferase activity driven by a TNF promoter construct. mTAL cells were cotransfected with either 1 \(\mu\)g/ml of pGV-B2-TNFprom or pGV-B2 (control construct lacking TNF promoter) and 1 \(\mu\)g/ml pae\textsubscript{c}-
\beta-galactosidase in the presence of 10 \(\mu\)l of lipofectamine reagent and 990 \(\mu\)l OPTI-MEM for 4 h at 37\textdegree C and 5% CO\textsubscript{2}. Transcriptional activity was assessed in cells that were exposed to media (control: 0.42 mM CaCl\textsubscript{2}) or additional amounts of CaCl\textsubscript{2} by using a luciferase assay system (Promega), and data were expressed as the increase of activation of normalized luciferase activity (luciferase/\(\beta\)-galactosidase) in cells treated with Ca\textsuperscript{2+} divided by the normalized activity in control cells. Inset: transfection with pGV-B2-TNFprom and incubation after addition of media or 1.0 mM CaCl\textsubscript{2}. For each condition, an additional 0.42 mM CaCl\textsubscript{2} was present in the media. \(\ast P < 0.05\); \(n = 6\).

Fig. 4. Effects of anti-TNF on Ca\textsuperscript{2+}-mediated PGE\textsubscript{2} production. mTAL cells were pretreated for 15 min with a neutralizing concentration of anti-TNF antibody and then treated with 1.2 mM CaCl\textsubscript{2} for 9 h. PGE\textsubscript{2} levels in supernatants were measured by ELISA. For each condition, an additional 0.42 mM CaCl\textsubscript{2} was present in the media. \(\ast P < 0.05\); \(n = 3\).

Fig. 5. Effects of anti-TNF on Ca\textsuperscript{2+}-mediated cyclooxygenase-2 (COX-2) protein expression. mTAL cells were pretreated with a neutralizing anti-TNF antibody and then treated with 1.2 mM CaCl\textsubscript{2} for 9 h. COX-2 protein levels were determined by Western blot analysis, and the blots were stripped and reprobed with an antibody against \(\beta\)-actin to correct for variations in sample loading. This is a representative figure from 4 similar experiments. For each condition, an additional 0.42 mM CaCl\textsubscript{2} was present in the media.
PKC contributes to Ca\textsuperscript{2+}-mediated TNF production. Direct activation of PKC enhances TNF production (35), and activation of the CaR was reported to enhance PKC activity (4). Thus the role of PKC in Ca\textsuperscript{2+}-mediated TNF production was determined. Cells were pre-treated with varying concentrations (0.25–1 \textmu M) of bisindolylmaleimide I, a selective PKC inhibitor (29), for 15 min and then challenged with 1.2 mM CaCl\textsubscript{2} for 9 h. Ca\textsuperscript{2+}-mediated TNF production was reduced by 52 and 73\% after treatment with 0.25 and 0.5 \textmu M bisindolylmaleimide I, respectively, suggesting that an increase in PKC activity after activation of the CaR contributes to TNF production (Fig. 6).

PKC regulates PGE\textsubscript{2} production and COX-2 protein expression. We previously demonstrated that PMA, a direct PKC activator, increased COX-2 mRNA accumulation, protein expression, and PGE\textsubscript{2} production (8). As the CaR activation also enhances PKC activity (4), COX-2 protein expression and PGE\textsubscript{2} synthesis were evaluated in cells challenged with Ca\textsuperscript{2+} in the absence or presence of bisindolylmaleimide I. Analysis of PGE\textsubscript{2} levels by ELISA revealed that mTAL cells produced 52.13 \pm 8.7 pg PGE\textsubscript{2}/\mu g protein after challenge with 1.2 mM CaCl\textsubscript{2} for 9 h, approximately a sixfold increase compared with basal levels of PGE\textsubscript{2} (Fig. 7). Basal levels of PGE\textsubscript{2} were not affected by bisindolylmaleimide I. However, PGE\textsubscript{2} synthesis induced by Ca\textsuperscript{2+} was inhibited by >70\% in the presence of 0.5 \textmu M bisindolylmaleimide I. These data suggest that basal production of PGE\textsubscript{2} in mTAL cells, which we previously showed was COX-1 dependent (8), is not PKC dependent. In contrast, CaR-mediated PGE\textsubscript{2} synthesis is PKC dependent. Because PGE\textsubscript{2} production in response to CaR activation is COX-2 dependent (31), it is not unexpected that there was a concomitant increase in COX-2 expression after challenge with Ca\textsuperscript{2+} (Fig. 8). Bisindolylmaleimide I attenuated the Ca\textsuperscript{2+}-dependent COX-2 protein expression, suggesting that increased PKC activity after CaR activation contributes to COX-2 expression in mTAL cells (Fig. 8).

DISCUSSION

We have demonstrated that mTAL cells in primary culture produced TNF after activation of the CaR, the first demonstration of what may be the basis of an important renal mechanism that regulates salt and water excretion (23, 24). Exposure of cells to CaCl\textsubscript{2} enhanced TNF production in a time- and concentration-dependent manner. Moreover, the CaR-selective agonist poly-L-arginine also increased TNF production, and the response to Ca\textsuperscript{2+} was increased by overexpression of the CaR. An increase in TNF gene transcription contributed to elevated levels of TNF subsequent to exposure to Ca\textsuperscript{2+}, and the increase in TNF synthesis contributes, by means of an autocrine mechanism, to...
enhanced COX-2 expression and PGE<sub>2</sub> synthesis in response to CaR activation. Expression of a functional CaR has been demonstrated in the mouse thick ascending limb (22). Exposure of cultured rat mTAL cells to increasing concentrations of Ca<sup>2+</sup> caused a dose-dependent increase in Ca<sup>2+</sup>, suggesting that a functional CaR was present in primary cultured mTAL cells (32). The CaR-mediated increases in TNF and PGE<sub>2</sub> production, as well as the increase in COX-2 protein expression, were PKC dependent. The present study also demonstrates that the CaR regulates COX-2 expression in the mTAL by TNF-dependent and -independent mechanisms.

The CaR is a G protein-coupled receptor that has three major domains: a large (613-amino acid) extracellular NH<sub>2</sub> terminus, a 250-amino acid domain with seven membrane-spanning segments, and a 222 amino acid cytoplasmic COOH-terminal domain. The extracellular domain has several regions rich in negatively charged amino acids, which could bind Ca<sup>2+</sup> and other cationic ligands (3, 4). Expression studies using Xenopus laevis oocytes injected with bovine parathyroid RNA demonstrated that exposure of oocytes to Ca<sup>2+</sup>-activated phospholipase C and increased intracellular levels of IP<sub>3</sub> and DAG (2). IP<sub>3</sub> triggers Ca<sup>2+</sup> release from the intracellular stores, and DAG activates PKC activity. These responses also may be elicited by other polyvalent CaR agonists, such as poly-L-arginine (11).

Although the precise signaling mechanisms that regulate the expression of TNF and COX-2 in mTAL cells have not yet been determined, the contribution of a PKC-dependent pathway indicates some similarity of the CaR expressed on cultured mTAL cells to those reported for several other cell types (37). The highest levels of the CaR are expressed on the basolateral surface of the cTAL, which reabsorbs divervalent minerals in a regulated manner (25). The CaR is also expressed on the basolateral side of mTAL cells and the apical membrane of the proximal tubule and inner medullary collecting duct. The basolateral localization of the CaR in the mTAL permits activation in response to alterations in interstitial and/or systemic levels of Ca<sup>2+</sup>. Accordingly, the CaR in the mTAL may be differentially activated according to the Ca<sup>2+</sup> concentration gradient that exists along the loop of Henle; the mean Ca<sup>2+</sup> concentration in tubular fluid vs. glomerular fluid was 2.8 at the papillary tip, whereas in the early distal tubule it was <1 (28). Thus, although the physiological role of the CaR includes the regulation of Ca<sup>2+</sup> homeostasis, expression of this receptor in the mTAL presumably contributes to the regulation of divalent cation, electrolyte, and water reabsorption (27), which affect extracellular fluid volume.

PKC activation was reported to induce TNF production in certain cell types (10, 19). Because activation of the CaR may enhance PKC activity (16), we hypothesized that TNF production in mTAL cells may be increased after CaR activation. Production of TNF in most cell types is low or absent before cellular stimulation. However, diverse extracellular stimuli, including exposure to Ca<sup>2+</sup> ionophore, antigen, virus infection, and LPS, can induce TNF gene expression. Several renal cell types can produce TNF, including proximal and mTAL tubular epithelial cells, as well as mesangial cells (1, 15, 20, 38). TNF may subserve several functions in the kidney, including regulation of pathophysiological events associated with inflammatory diseases in the kidney (5, 17, 36). TNF also may contribute to the chronic tubular injury associated with hypercalcemia. These findings suggest a role for TNF in renal physiological/pathophysiological mechanisms and raise the distinct possibility that other cytokines may contribute to a network of effects in the kidney.

Transcriptional regulation of TNF production by Ca<sup>2+</sup> was assessed by transfecting mTAL cells with a rat TNF promoter construct that drives expression of a luciferase reporter gene in the pGV-B2 plasmid. The significant increase in luciferase activity suggests that increased TNF promoter activity by means of CaR activation contributed, at least in part, to the increased synthesis of this cytokine. Transcription of the TNF gene does not require de novo protein synthesis and depends on the recruitment of transcription factors in a cell type-specific manner (30). Moreover, TNF gene transcription in T lymphocytes is characterized by formation of distinct enhancer complexes on the TNF promoter in response to different extracellular stimuli. For instance, distinct sets of TNF promoter elements were required for induction of TNF gene transcription by T cell receptor activation, Ca<sup>2+</sup> influx, or virus infection (7). Because activation of the CaR may initiate several signal transduction pathways in a cell type-specific manner, it is interesting to note that inhibition of PKC activity in mTAL cells attenuated TNF production. CaR overexpression experiments showed that the Ca<sup>2+</sup>-dependent TNF production was enhanced, supporting the contention that activation of the CaR leads to an increase in TNF production in the mTAL.

The CaR agonist poly-L-arginine increased TNF production, further supporting a role for the CaR in this regard. Although the precise mechanism and transcription factors that contribute to the increase of TNF by means of CaR activation in the mTAL are not known, Ca<sup>2+</sup> influx in T cells results in the recruitment of ATF-2/c-jun and NF-AT to the TNF promoter. Moreover, calcineurin phosphatase activity is required for full activation of the TNF gene in B cells, fibroblasts, and T cells. Thus the ability of CaR activation to increase intracellular concentrations of Ca<sup>2+</sup> may activate a Ca<sup>2+</sup>/calmodulin-dependent calcineurin pathway in mTAL cells. The role of these transcription factors and the contribution of PKC-independent mechanisms in mTAL cells have yet to be determined.

TNF has been shown to increase COX-2 protein expression in several cell types. We found that incubation of mTAL cells with rat recombinant TNF increased PGE<sub>2</sub> production by a COX-2-dependent mechanism (8). Pretreatment of cells with a COX-2 inhibitor prevented TNF-mediated inhibition of <sup>86</sup>Rb uptake, an in vitro correlate of natriuresis. This mechanism also was demonstrated for endogenous TNF as LPS-induced inhibition of <sup>86</sup>Rb uptake was abolished in the
presence of an anti-TNF antibody, suggesting that TNF produced by the mTAL acted in an autocrine manner to inhibit 86Rb uptake (6). In the present study, a neutralizing anti-TNF antibody significantly reduced Ca2+-dependent PGE2 production, suggesting that TNF acts in an autocrine manner in the mTAL to upregulate local PGE2 production. However, it is likely that a TNF-independent component is also initiated on CaR activation, because there still were significant levels of PGE2 synthesis in the absence of TNF. As the CaR increases phospholipase A2 activity in the mTAL (33), we hypothesize that the residual PGE2 production may reflect metabolism of arachidonic acid via COX-1. Moreover, it is likely that multiple signaling pathways contribute to COX-2 expression in mTAL cells. To our knowledge, this study is the first demonstration that TNF production can be induced after activation of the CaR. Regulation of TNF production by Ca2+ may be the basis of a mechanism in which COX-2 protein expression and activity are modulated in the mTAL. Disturbances in renal concentrating ability, water intake, and loop of Henle function have been associated with hypercalcemia in humans and experimental animals (24); TNF may be linked to inhibition of NaCl reabsorption by the TAL in response to hypercalcemia (6). Indeed, a necessary link in the mechanism by which hypercalcemia induces polyuria-natriuresis appears to be TNF production by the mTAL, with expression of COX-2 and synthesis of PGE2.

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