Curcumin and enalapril ameliorate renal failure by antagonizing inflammation in 5/6 nephrectomized rats: role of phospholipase and cyclooxygenase

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Curcumin and enalapril ameliorate renal failure by antagonizing inflammation in 5/6 nephrectomized rats: role of phospholipase and cyclooxygenase. Am J Physiol Renal Physiol 302: F439–F454, 2012. First published October 26, 2011; doi:10.1152/ajprenal.00356.2010.—Previously, we showed that curcumin prevents chronic kidney disease (CKD) development in 5/6 nephrectomized (Nx) rats when given within 1 wk after Nx (Ghosh SS, Massey HD, Krieg R, Fazelboly ZA, Ghosh S, Sica DA, Fakhry I, Ghosh S, and Gehr TW. Am J Physiol Renal Physiol 296: F1146–F1157, 2009). To better mimic the scenario for renal disease in humans, we began curcumin and enalapril therapy when proteinuria was already established. We hypothesized that curcumin, by blocking the inflammatory mediators TNF-α and IL-1β, could also reduce cyclooxygenase (COX) and phospholipase expression in the kidney. Nx animals were divided into untreated Nx, curcumin-treated, and enalapril-treated groups. Curcumin (75 mg/kg) and enalapril (10 mg/kg) were administered for 10 wk. Renal dysfunction in the Nx group, as evidenced by elevated blood urea nitrogen, plasma creatinine, proteinuria, segmental sclerosis, and tubular dilatation, was comparably reduced by curcumin and enalapril, with only enalapril significantly lowering blood pressure. Compared with controls, Nx animals had higher plasma/kinney TNF-α and IL-1β, which were reduced by curcumin and enalapril treatment. Nx animals had significantly elevated kidney levels of cytosolic PLA2, calcium-independent intracellular PLA2, COX 1, and COX 2, which were comparably reduced by curcumin and enalapril. Studies in mesangial cells and macrophages were carried out to establish the in vivo increase in PLA2 and COX were mediated by TNF-α and IL-1β and that curcumin, by antagonizing the cytokines, could significantly reduce both PLA2 and COX. We conclude that curcumin ameliorates CKD by blocking inflammatory signals even if it is given at a later stage of the disease.

CKD; cytokines; nephrectomy; remnant

Curcumin is the active ingredient in the traditional herbal remedy and dietary spice turmeric (Curcuma longa) and is undergoing clinical trials for various diseases including cancer, Alzheimer’s disease, and ulcerative colitis (25). It is suggested that many of the beneficial effects of curcumin relate to its ability to suppress both acute and chronic inflammation (4). In our earlier studies, we have shown that curcumin inhibits TNF-α-dependent NF-κB activation and blocks the TNF-α-mediated downregulation of peroxisome proliferator-activated receptor γ (PPARγ) in mesangial cells (19). A similar effect of curcumin on PPARγ and NF-κB has been shown in hepatic stellate cells, further supporting the prominent anti-inflammatory effect of curcumin (60). However, variable absorption and poor bioavailability due to first-pass metabolism have been an issue with curcumin use in humans (3).

Curcumin prevents cardiac hypertrophy in salt-sensitive Dahl rats (39). It has been shown to block fibrosis in nephritic rats by inducing hemoxyenase (15) and attenuates carbon tetrachloride-induced liver fibrosis in rats (14), suggesting that at least in murine models the absorption of curcumin to reach a therapeutic endpoint is not a significant problem. In our earlier studies, we have shown that curcumin alleviates chronic renal failure (CRF) in the 5/6 nephrectomized (Nx) rat model when curcumin therapy was started within 1 wk of Nx and before the onset of proteinuria. Based on these positive findings with curcumin when preemptively given, we sought to determine whether curcumin could ameliorate CRF when treatment is initiated at a later stage of the disease, in that therapies for renal failure are generally given as the disease is already in progress.

Patients with CKD have higher circulating levels of proinflammatory cytokines (43, 57). Similarly, high expression of cytokines such as IL-1β and TNF-α has been observed in rat models of CRF (21, 41). Anti-inflammatory agents and molecules which antagonize cytokines are known to ameliorate CRF (21, 45). In uremia, deterioration of renal function may be one of the most important factors associated with significant increases in TNF-α and IL-1β activity. Both cytokines provide a rapid form of host defense against infection; however, when present in excess, they can prove harmful (55). Proinflammatory cytokines such as TNF-α and IL-1β can act as toxins participating in uremic complications (53), generate reactive oxygen species (ROS), NF-κB, and IL-6 (31, 32), and attract macrophages to inflammatory sites (19). We further speculated by blocking the actions of TNF-α and IL-1β that we will be able to reduce renal dysfunction even when the therapy is started after the onset of proteinuria. We also used enalapril as a positive control for these studies because ANG II is a known inflammatory mediator and angiotensin-converting enzyme inhibitors (ACEI) are known to ameliorate both experimental and human renal failure (46, 56).

Furthermore, TNF-α and IL-1β can induce the PLA2 family, which, in kind, are known to stimulate inflammatory mediator production (34). Studies in cytosolic PLA2 (cPLA2) knockout animals have shown that this lipid-metabolizing enzyme has been implicated in many pathophysiological processes such as arthritis and also in various kidney disorders including models of ischemic injury, diabetic nephropathy, and anti-thy1 glomerulonephritis (7, 8). Originally, calcium-independent intracellular PLA2 (iPLA2) was suggested to mediate phospholipid remodeling and was thought not to play as significant a role as its counterpart, cPLA2. However, later studies have shown that iPLA2 and cPLA2 regulate monocyte migration from different

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intracellular locations, with iPLA$_2$ acting as a critical regulator (38). In cell lines, curcumin has been shown to inhibit cPLA$_2$, and both COX 1 and COX 2 isoforms (27). Therefore, in these studies we examined the contribution of the lipid-metabolizing enzymes cPLA$_2$, iPLA$_2$, and COX isoforms to outcomes in Nx animals with already established renal failure.

**MATERIALS AND METHODS**

Curcumin was purchased from Biomol. Phosphorylated cPLA$_2$ was obtained from Cell Signaling (Danvers, MA), and other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University. Rats were divided into four groups with six animals in each group. The control group underwent sham surgery, and three other groups underwent Nx by the procedure described below.

**Surgery.** CRF was induced in rats by the method published previously (19). Briefly, 5⁄6 nephrectomy of rats, weighing between 150 and 200 g, was performed using sterile techniques. All operations were carried out under isoflurane anesthesia. A left-flank incision was made, and the left kidney was exposed. The renal artery was temporarily occluded, and the upper and lower thirds of the kidney were ligated and excised. Bleeding was controlled by manual compression until it stopped; thus, one-third of the mass of the left kidney remained. The muscle and skin incisions were sutured with polypropylene suture. The animals were returned to the vivarium to recover. One week later, a right-flank incision was made, the renal vessels and ureter were tied, and the right kidney was excised. The treatments were started 6 wk after the second surgery and continued for 16 wk. At week 17, the animals were euthanized. Suspensions of curcumin and enalapril were made in 0.5% carboxymethylcellulose (CMC; curcumin is nonpolar and must be suspended in CMC), and the sham-operated control received volume-matched 0.5% CMC. Due to the instability of curcumin in an aqueous system, the compound was made fresh and given within 10 min of preparation. The animals were divided into groups (Fig. 1) as follows.

**Group I:** sham operated and received volume-matched 0.5% CMC 6 wk after the second Nx.

**Group II:** untreated Nx animals euthanized 6 wk after the second Nx.

**Group III:** untreated Nx animals euthanized at week 17 after the second Nx.

**Group IV:** curcumin-treated (75 mg/kg) Nx animals; treatment started 6 wk after second Nx, and animals were euthanized at week 17.

**Group V:** enalapril-treated (75 mg/kg) Nx animals; treatment started 6 wk after second Nx and animals were euthanized at week 17.

Following death, blood and tissue were collected for analyses.

**Blood Urea Nitrogen and Creatinine**

Blood urea nitrogen (BUN) and creatinine were measured by a NOVA16 autoanalyzer (NOVA Biomedical, Waltham, MA).

**Determination of Kidney ANG II and Serum ANG II, TNF-α, and IL-1β**

Kidney and serum ANG II were measured as described earlier (16). At the time of death, trunk blood was obtained from the aorta and placed in prechilled tubes containing protease inhibitors. The serum was separated by centrifugation at 4°C and stored at −70°C. Kidneys were homogenized within 30 s in prechilled methanol in a glass tissue grinder. The homogenate was centrifuged at 4°C for 30 min at 1,200 rpm, and the supernatant was dried in a vortex evaporator (HBI, Lenexa, KS). The dried residue was reconstituted with 50 mmol/l phosphate buffer, pH 7.4. Plasma and kidney ANG II were processed for radioimmunoassay according to a published procedure (ALPCO Diagnostics, Salem, NH). In brief, the samples were extracted on a phenylsilysilica column with methanol, and the eluant was dried and processed for radioimmunoassay using a specific antibody to ANG II (ALPCO). The serum TNF-α and IL-1β were determined using a rat TNF-α ELISA kit (ALPCO) and rat IL-1β ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions.

**Longitudinal Measurement of Arterial Pressure by Tail Plethysmography**

Arterial pressure (BP) was determined by tail plethysmography as previously described (18) using the CODA 2 system (Kent Scientific, Torrington, CT). CODA 2 utilizes volume pressure recording sensor technology to measure tail BP. This is a computerized, noninvasive tail-cuff acquisition system, which can simultaneously measure systolic, diastolic, and mean arterial pressure without operator intervention. Training of the rats for the measurement of BP was started 2 wk after Nx. The rats were kept in a holder for a 5- to 10-min period, and during this period 25 sequential readings were obtained. Readings within a range of 10 mmHg were averaged. In this study, we report the mean systolic BP pressure of each group.

**Histology**

Animals from each group were studied for histological changes in the kidney. A portion of the kidney was cut and fixed in 10% buffered formalin for light microscopy. The basic scoring system, described previously (19), quantified the fraction of total glomeruli showing global and/or segmental sclerosis, the percentage of tubules showing dilatation, and epithelial hyperplasia. A minimum of 50 glomeruli/animal were scored by an observer blinded to the origin of the tissue. Sections were cut at 2-μm thickness and stained with periodic acid-Schiff.

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**Fig. 1.** Experimental protocol. Arrows indicate time points for treatment, performance of surgical procedures, various groups, and time of death. Nx, nephrectomized animals.

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Homogenization

Kidney cytosol. Each kidney was cut and immediately frozen in liquid nitrogen and kept at −70°C until use. The frozen kidney was ground to a powder and then mixed in ice-cold NE-PER buffer (Thermo Fisher Scientific, Rockford, IL) and protease and phosphatase inhibitors. The kidney was homogenized in an ice-chilled Dounce homogenizer at 4°C, and the cytosolic and nuclear extract were isolated according to the manufacturer’s protocol. The cytosolic extracts were aliquoted and stored at −70°C until use. The nuclear extracts which were not utilized in this study were aliquoted and stored at −70°C.

Immunoblotting

Kidney homogenates and cytosolic (75–100 μg total protein) and nuclear extracts (50 μg total protein) were separated on a 4–20% SDS-PAGE gel, and proteins were transferred to a polyvinylidene difluoride membrane as described before (19). After being briefly washed in phosphate-buffered saline containing 1% Tween 20 (PBS-T) and blocked in 5% nonfat dry milk, blots were incubated with appropriate antibodies in 5% nonfat dry milk overnight at 4°C. After being washed three to five times in PBS-Triton X-100, blots were subsequently incubated with secondary antibody appropriately diluted in 5% nonfat dry milk for 1 h at room temperature. After being washed three to five times in PBS, blots were developed using Lightning Chemiluminescence Reagent Plus and exposed to X-rays.

Cell Culture

Mesangial cells. Rat mesangial cells were obtained from American Type Culture Collection (Manassas, VA) and grown as previously described (17) in Ham’s F12 and DMEM with 15% FBS. For transfection studies, cells were plated in 24-well plates. For other studies, they were plated in 35-mm plates.

Mesangial cells (~90–95% confluent) were incubated with serum-free media for 24 h to arrest and synchronize cell growth. For curcumin dose-response studies, the cells were treated with varying concentrations of curcumin (5–20 μM) for 24 h. For time course studies, cells were treated with cytokines and curcumin for 0–24 h. Curcumin (5–20 μM) was dissolved in 15 μl ethanol. TNF-α and IL-1β were added 3 h after curcumin. Cells were harvested for protein in ice-cold lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors, 24 h after the addition of curcumin.

Macrophages. For induction of macrophages, rats were injected intraperitoneally with 1 ml of 3% thioglycollate broth, and peritoneal exudate was extracted 2 days later. Peritoneal macrophages were obtained as previously described (63) and plated in six-well tissue culture plates in RPMI supplemented with 10% FBS. Nonadherent cells were removed after 24 h. They were treated with cytokines in the presence or absence of curcumin as described above (see Mesangial cells).

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from kidneys with the RNeasy Mini Kit as described before (18). Briefly, 2 μg of total RNA were reverse transcribed with the Thermoscript RT-PCR System (Invitrogen), and first-strand cDNA was used to perform real-time PCR using the Stratagene Mx3000p real-time PCR system with TaqMan Gene Expression Assays for TNF-α, IL-1β, and β-actin obtained from Applied Biosystems (Foster City, CA). The amount of mRNA was calculated by the ΔCT method and normalized to β-actin.

Statistical Analysis

Statistical comparisons among groups were performed using ANOVA followed by Tukey’s multiple comparison test. Groups were considered to be significantly different at P ≤ 0.05.

RESULTS

Effect of Curcumin and Enalapril on Body Weight, Kidney Weight, and Kidney Weight/Body Weight Ratio

Weight loss is a problem with CKD, and in this study we found that untreated Nx animals had a significant loss in body weight. As shown in Fig. 2A, the body weight of untreated

![Figure 2A](image-url)  
**Fig. 2A.** Comparison of body weight, kidney weight, and kidney weight/body weight ratio in sham-operated (control), untreated 6-wk Nx (Nx 6wk) and untreated 16-wk Nx (Nx 16wk) animals, and curcumin- and enalapril-treated animals. The body weight, kidney weight, and kidney weight/body weight ratio are shown. Values are means ± SD; n = 5 animals/group. *P < 0.05, †P < 0.01 compared with Nx.
6-wk Nx animals and 16-wk Nx animals were 14 ($P < 0.05$) and 29% ($P < 0.01$) lower than control, respectively. Although at the end of 16 wk curcumin- and enalapril-treated animals weighed significantly more than the 16-wk Nx animals ($P < 0.05$), their weights were not significantly different from the 6-wk group and were significantly lower than the control ($P < 0.05$). The remnant kidney weights of the Nx animals were equivalent to the left kidney weights of sham-operated animals, suggesting hypertrophy of the remnant kidney (Fig. 2B). This is further emphasized by the fact that the left kidney weight/body weight ratio of the control was significantly lower than the remnant kidney weight/body weight ratio of 16-wk Nx animals ($P < 0.01$) but not different from 6-wk Nx animals or treated animals (Fig. 2C).

**Effect of Curcumin and Enalapril on BP, Proteinuria, Plasma Creatinine, and BUN**

Subtotal nephrectomy resulted in renal dysfunction, as evidenced by both a gradual increase in proteinuria (Fig. 3A) and elevated BUN and plasma creatinine at the end of 16 wk (Fig. 3, C and D, respectively). The level of proteinuria of Nx animals was significantly higher than that of controls from 4 wk onward (Fig. 3A). Nx animals were divided into four groups as described earlier (Fig. 1), and at the beginning of week 6 two groups of Nx animals were treated with either curcumin or enalapril, and the data were compared with those from untreated 6- and 16-wk Nx animals. In the first 2 wk of treatment, no significant improvement in proteinuria was observed in the treatment groups. However, by week 12 curcumin- and enalapril-treated animals showed a 21 ($P < 0.05$) and 27% ($P < 0.05$) decrease, respectively, in proteinuria compared with values from week 6. By the end of week 16, treatment with curcumin and enalapril decreased proteinuria by 44 ($P < 0.01$) and 40% ($P < 0.01$), respectively. The change in proteinuria in curcumin and enalapril cohorts was not significantly different at any point of time. BUN and plasma creatinine measurements made at the end of 16 wk showed that these surrogate markers for the level of renal function were 3.0- and 4.3-fold higher, respectively, in the untreated Nx animals than in the vehicle-treated controls. Curcumin was as effective as enalapril in limiting the rise in both BUN and creatinine otherwise seen in untreated 16-wk Nx animals ($P < 0.01$). However, both creatinine and BUN values of curcumin- and enalapril-treated animals were significantly higher than the control ($P < 0.05$). The creatinine and BUN values of 6-wk Nx animals were also significantly higher than in the controls ($P < 0.05$) (Fig. 3, C and D). Although the creatinine values of 6-wk Nx animals were 1.3- and 1.5-fold higher than the curcumin- and enalapril-treated groups, respectively, the difference was not statistically significant. A similar difference was seen in BUN levels of 6-wk Nx animals and in curcumin (14% lower) and enalapril-treated (10% lower) animals, but they were not statistically different from untreated 6- and 16-wk Nx animals.
statistically significant. Systolic BP did not change during the first 2 wk, but steadily rose with time (Fig. 3B), with values at 6 wk significantly higher in all Nx animals compared with controls (P < 0.01). The effect of enalapril in curtailting the rise in BP was evident within 2 wk of treatment (P < 0.01). Enalapril treatment effectively blocked the rise in systolic BP until the end of the study (P < 0.01). In the curcumin group, the BP was similar to Nx animals until 16 wk, when it was significantly reduced (P < 0.05), although not to the degree that it was in enalapril-treated animals. It is to be noted that the systolic BP (4, 6, and 8 wk) of Nx animals measured in this study was 8–10 mmHg lower than our earlier study (19), and we are unable to explain the reason for this difference. The reduction in proteinuria in the curcumin-treated group occurred independently of BP.

**Histology**

Histological examination of the kidney supported the biochemical findings of renal failure in Nx animals, findings that were significantly improved by curcumin and enalapril. Figure 4 shows a representative example of the histological changes seen in each group. The results are quantified in a summary fashion in Table 1. Control animals did not show any significant glomerular damage, and 6-wk Nx animals had 13.4 ± 8.9% segmental sclerosis. This was significantly lower than in curcumin- and enalapril-treated animals (P < 0.05). Significant variations in global sclerosis (7.5 ± 5.6%) and tubular dilatation (10.4 ± 9.5%) were observed in 6-wk Nx animals, and they were not significantly different from the treated groups. In contrast, untreated Nx animals had 45.1 ± 8.5% segmentally sclerosed glomeruli and 30.1 ± 8.1% globally sclerosed glomeruli. Segmental and global sclerosis in curcumin- and enalapril-treated animals were 27.3 ± 5.5% (P < 0.05 compared with Nx) and 30.1 ± 3.5%, respectively (P < 0.05, compared with Nx). Compared with Nx animals, curcumin- and enalapril-treated animals had significantly fewer glomeruli that were globally sclerosed (P < 0.01). The global sclerosis in curcumin- and enalapril-treated animals were 6.9 ± 3.9 and 5.3 ± 2.3%, respectively. There was no difference between curcumin- and enalapril-treated animals in the observed level of either global or segmental sclerosis. In addition to pronounced glomerular sclerosis, severe tubulointerstitial dilatation was observed in the untreated Nx rats. Untreated Nx rats had 42.3 ± 5.1% dilated tubules, a finding which was significantly reduced to 4.3 ± 1.1 (P < 0.001 compared with Nx) and 5.1 ± 0.9% (P < 0.001 compared with Nx) in curcumin- and enalapril-treated rats, respectively. Tubular dilation in curcumin- and enalapril-treated groups was not significantly different. In addition, untreated Nx animals had moderate renal tubular epithelial hyperplasia, which was not observed in curcumin- and enalapril-treated animals.

**Effect of Curcumin and Enalapril on TNF-α and IL-1β**

The plasma TNF-α concentrations (73.8 ± 12.1 pg/ml) of untreated Nx animals were twofold higher than those of controls. Curcumin and enalapril treatment reduced TNF-α levels by 27 (P < 0.05) and 37% (P < 0.01), respectively (Fig. 5A). The kidney TNF-α mRNA of Nx animals was about fourfold lower than controls. Curcumin vs. Nx-6 wk, P < 0.05 Enalapril vs. Nx-6 wk, P < 0.05 Curcumin vs. Nx-6 wk, P < 0.05 Enalapril vs. Nx-6 wk, P < 0.05 Curcumin vs. Nx-16 wk, P < 0.01 Enalapril vs. Nx, P < 0.01 Curcumin vs. Nx-16 wk, P < 0.001 Enalapril vs. Nx-16 wk, P < 0.001

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**Table 1. Differences in tubular dilatation, global, and segmental sclerosis between 6- and 16-wk % nephrectomized and curcumin- and enalapril-treated cohorts**

<table>
<thead>
<tr>
<th></th>
<th>Nx-6 wk</th>
<th>Nx-16 wk</th>
<th>Curcumin</th>
<th>Enalapril</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Segmental sclerosis</td>
<td>13.4 ± 8.9</td>
<td>45.1 ± 8.5</td>
<td>27.3 ± 5.5</td>
<td>30.1 ± 3.5</td>
<td>Curcumin vs. Nx-6 wk, P &lt; 0.05 Enalapril vs. Nx-6 wk, P &lt; 0.05 Curcumin vs. Nx-6 wk, P &lt; 0.05 Enalapril vs. Nx-6 wk, P &lt; 0.05 Curcumin vs. Nx-16 wk, P &lt; 0.01 Enalapril vs. Nx, P &lt; 0.01 Curcumin vs. Nx-16 wk, P &lt; 0.001 Enalapril vs. Nx-16 wk, P &lt; 0.001</td>
</tr>
<tr>
<td>% Global sclerosis</td>
<td>7.5 ± 5.6</td>
<td>30.1 ± 8.1</td>
<td>6.9 ± 3.9</td>
<td>5.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>% Tubular dilatation</td>
<td>10.4 ± 9.5</td>
<td>42.3 ± 5.1</td>
<td>4.3 ± 1.1</td>
<td>5.1 ± 0.9</td>
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Values are means ± SD; n = 5 animals/group. Nx, nephrectomized animals. The histological changes in the curcumin and enalapril group were not significantly different.
higher \((P < 0.01)\) than the control and as shown in Fig. 5B. Enalapril and curcumin treatment reduced TNF-\(\alpha\) message by almost 3 \((P < 0.01)\)- and 1.5-fold, respectively \((P < 0.05)\). The mean plasma concentration of TNF-\(\alpha\) in untreated Nx rats was fourfold higher than the control \((P < 0.01)\) (Fig. 5D), and the elevated levels of this cytokine were reduced by both curcumin and enalapril by almost twofold \((P < 0.05)\). It is to be noted that although curcumin effectively reduced plasma and kidney TNF-\(\alpha\) in Nx animals, enalapril had a more significant effect on this cytokine. However, both lowered IL-1\(\beta\) to a similar degree.

**Effect of Curcumin and Enalapril on ANG II**

Curcumin and enalapril blocked the increase in TNF-\(\alpha\) and IL-1\(\beta\) in the plasma and kidney of Nx animals, but the mechanism for this is not clear. TNF-\(\alpha\) production is known to be stimulated by ANG II (26), and the ANG II antagonist olmesartan has been shown to block the increase in IL-1\(\beta\) in experimental autoimmune myocarditis (62). Thus we investigated whether curcumin had any effect on ANG II levels in these animals. As shown in Fig. 6, Nx animals did not have any increase in plasma ANG II but we did see a small but significant increase in ANG II levels in the kidney \((P < 0.05)\). Enalapril significantly reduced both plasma and kidney ANG II in contradistinction to curcumin, which had no effect on plasma or tissue ANG II levels.

**Effect of Curcumin and Enalapril on cPLA2 and iPLA2**

The PLA2 family hydrolyzes arachidonic acid from the sn-2 position of glycerophospholipids, thereby providing the substrate necessary for COX 1 and COX 2. The cPLA2s are large cytosolic proteins, ranging from 61 to 114 kDa (50). Our PLA2 antibody did not show any cross-reactivity with any protein higher than 84 kDa, suggesting it is a group IVA cPLA2. The catalytic mechanism of group IVA cPLA2 involves a Ser/Asp dyad, and phosphorylation of this site generates the active protein (50). The ratio of phosphorylated cPLA2 to nonphosphorylated PLA2 from the kidney cytosol of untreated animals (Fig. 5A) was more than twofold higher than the control group \((P < 0.001)\). Both curcumin and enalapril treatment were equally effective in blocking the increased phosphorylation and, as shown in Fig. 7A, the ratio of phosphorylated to
nonphosphorylated cPLA₂ of the treated groups was significantly lower ($P < 0.01$) than in the Nx cohort and not significantly different from controls.

Murine iPLA₂ (group VIA PLA₂) ranges from 84 to 146 kDa (55); although known to release arachidonic acid, it plays a more important role in releasing other fatty acids and in cell signaling (28). No phosphorylation consensus sequences, however, have been described in iPLA₂ (5). As shown in Fig. 7B, compared with controls the average increase in iPLA₂ in the kidney of Nx animals was $\sim$1.6-fold ($P < 0.01$). This increase in iPLA₂ was significantly reduced by curcumin ($P < 0.05$) but not by enalapril. The iPLA₂ antibody used in this study detected a protein of 84-kDa molecular mass. There are at least three isoforms of iPLA₂ (VIA-1, VIA-2, and VIB) that have a molecular mass of 84 kDa, generated due to alternate splicing (5, 50). In this study, we could not distinguish the specific isoform(s) in play.

**Effect of Curcumin and Enalapril on COX 1 and COX 2**

Arachidonic acid released by PLA₂s is converted to prostanoids by COX 1 and COX 2. Both COX 1 and COX 2 play an important role in physiological and pathophysiological processes including renal blood flow and inflammation (23), and COX 1 and COX 2 upregulation has been observed in Nx rats (58). In our earlier study, we had seen a significant influx of mesangial cells and macrophages in the damaged kidney (19), and these macrophages can contribute to the release of inflammatory mediators. In addition, multiple cytokines are released in both experimental and clinically inflammatory disease such as CKD. We wanted to investigate whether interaction between the cytokines could potentiate the expression of the enzymes. We used mesangial cells and macrophages to study the interaction and investigate the effect of curcumin because these cells express isoforms of both COX and PLA₂.

**Rationale for In Vitro Experiments with Mesangial Cells**

These in vivo experiments have shown that curcumin counteracts the elevation of TNF-α and IL-1β and blocks the increase in PLA₂ and COX isoforms. Although it suggests that curcumin has an anti-inflammatory role, it does not specifically demonstrate the mechanism of the antagonistic effect of curcumin. In the 16-wk Nx animals, we saw significantly increased levels of cytokines (TNF-α and IL-1β). We also observed augmented expression of the inflammatory enzymes PLA₂ and COX. The increased expression of PLA₂ and COX in vivo could be due to multiple factors, including the enhanced levels of cytokines. To demonstrate that TNF-α and IL-1β were playing a role in the induction of these enzymes and to prove curcumin can block them, we needed an in vitro model. In our earlier study, we had seen a significant influx of macrophages in the damaged kidney (19), and these macrophages can contribute to the release of inflammatory mediators. In addition, multiple cytokines are released in both experimental and clinically inflammatory disease such as CKD. We wanted to investigate whether interaction between the cytokines could potentiate the expression of the enzymes. We used mesangial cells and macrophages to study the interaction and investigate the effect of curcumin because these cells express isoforms of both COX and PLA₂.

**Effect of TNF-α and IL-1β on Expression of cPLA₂, iPLA₂, COX 1, and COX 2**

All PLA₂s (including cPLA₂ and iPLA₂) (22, 52) and both COX 1 and COX 2 are constitutively expressed in mesangial cells and macrophages (36, 55). Infiltrating macrophages can significantly contribute to the inflammatory milieu; therefore, cultured rat mesangial cells and rat peritoneal macrophages were used to investigate whether TNF-α and/or IL-1β could...
stimulate PLA2s and COX isoforms. Mesangial cells have previously been used as an in vitro model to investigate and explain the expression of various proteins mediated by TNF-α and IL-1β. However, concentrations of both molecules for in vitro experiments have ranged from 1 ng/ml to >20 ng/ml (40, 47, 48); therefore, we established a dose range for both biomolecules, which would produce significant expression of PLA2 and COX isoforms in mesangial cells. Mesangial cells were made quiescent by serum starving for 24 h and then were treated with TNF-α and IL-1β in media with 0.5% serum. We compared untreated mesangial cells (0.5% serum-containing media) with cells treated with TNF-α (2.5–10 ng/ml) or IL-1β (0.5–10 ng/ml) for 24 h and observed that the minimum concentration of TNF-α and IL-1β to elicit significant expression of cPLA2, iPLA2, and COX 2 were 10 and 5 ng/ml, respectively (data not shown); however, COX 1 was not induced by either cytokine in the dose range studied. We used the same concentrations of cytokines to stimulate the macrophages. We did a time course study (see Figs. 8–11) where mesangial cells and macrophages were incubated for up to 24 h with either TNF-α (10 ng/ml) or IL-1β (5 ng/ml) or a mixture of TNF-α (10 ng/ml) and IL-1β (5 ng/ml) and compared with cells which were not exposed to either cytokine for 24 h. As shown in Fig. 8, A and B, the ratio of phosphorylated to nonphosphorylated cPLA2 in TNF-α and IL-1β-treated cells were 1.7 (P < 0.05)- and 1.6-fold (P < 0.05) higher, respectively, in untreated mesangial cells. A similar increase was also observed in macrophages. At the end of 24 h, the increase in cPLA2 following TNF-α treatment was slightly less than threefold (P < 0.01) (Fig. 9A), and IL-1β-mediated activation was twofold (P < 0.01) (Fig. 9B). TNF-α and IL-1β also stimulated iPLA2 by 1.8 (P < 0.05)- and 2-fold (P < 0.05), respectively, in mesangial cells (Fig. 8, A and B). In macrophages, iPLA2 was activated 1.7-fold by TNF-α (P < 0.05) (Fig. 9A) and 2-fold by IL-1β (P < 0.05) (Fig. 9B). When mesangial cells and macrophages were exposed to both TNF-α (10 ng/ml) and IL-1β (5 ng/ml) (Figs. 8C and 9C), both cPLA2 and iPLA2 were significantly increased (P < 0.01); however, the more than fourfold increase in cPLA2 and more than threefold increase in iPLA2 were substantially greater than the individual cytokine effects of cPLA2 and iPLA2 on mesangial cells and macrophages (Figs. 8 and 9). Moreover, when the cytokines were given together, a significant increase in both cPLA2 and iPLA2 was observed as early as the 10-h time point in both mesangial cells and macrophages (Figs. 8C and 9C) a finding that was not seen when mesangial cells and macrophages were treated separately with TNF-α or IL-1β (Figs. 8, A and B, and 9, A and B).

Neither TNF-α nor IL-1β independently stimulated COX 1 in either mesangial cells or peritoneal macrophages (Figs. 10, A and B, and 11, A and B); however, when mesangial cells were treated with both cytokines, a significant stimulation of COX 1 was observed at 24 h (P < 0.05) (Fig. 10C). Surprisingly, we did not see similar stimulation of COX 1 in macrophages (Fig. 11C). We are unable to explain this discrepancy. Compared with the untreated cells, the mesangial cells treated for 24 h with TNF-α had a 1.5-fold increase in COX 2 expression (P < 0.05) (Fig. 10A), while the increase in macrophage COX 2 expression was 1.8-fold (P < 0.01). A similar increase in COX 2 expression in mesangial cells (1.6-fold; P < 0.05) and macrophages (1.8-fold; P < 0.01) was seen with IL-1β treatment (Figs. 10B and 11B). Compared with untreated cells, the combination of TNF-α and IL-1β augmented COX 2 expression by 2.5-fold in mesangial cells (P < 0.01) and 4.5-fold (P < 0.01) in macrophages (Figs. 11C and 12C). Although COX 2 expression in mesangial cells at 10 h was 1.4-fold higher than the untreated cells, it was not a statistically significant finding; however, in macrophages the cytokine mixture caused 2-fold stimulation at 10 h (P < 0.05).

These experiments suggest that TNF-α and IL-1β not only increase the expression of PLA2S and COX isoforms but in combination may have a synergistic, or strongly additive, effect on these lipid-metabolizing enzymes.

Effect of Curcumin on TNF-α- and IL-1β-Induced Expression of cPLA2, iPLA2, COX 1, and COX 2

Compared with controls, Nx animals had significantly higher levels of PLA2 and COX isoforms, and curcumin treatment arrested this increased expression. Our in vitro experiments provided evidence that TNF-α and IL-1β can increase the expression of both PLA2S and COX isoforms. To investigate whether curcumin could antagonize the effect of these cytokines on these lipid-metabolizing enzymes, mesangial cells were treated with 5–20 μM curcumin and 3 h later the cells were exposed to a combination of TNF-α (10 ng/ml) and IL-1β (5 ng/ml) for an additional 24 h. Curcumin concentrations of 10 and 20 μM inhibited cPLA2 by 36 (P < 0.05) and 66% (P < 0.01), respectively (Fig. 12A). A significantly decreased expression of iPLA2 (38%) was observed with 10 μM curcumin (P < 0.05), and 20 μM of curcumin reduced expression of iPLA2 by ~50% (Fig. 12B). Curcumin (10 μM) reduced expression of COX 1 and COX 2 by 1.7- and 1.4-fold, respectively (Fig. 12, C and D), but this did not reach statistical significance. Higher concentrations of curcumin (20 μM), however, significantly blocked cytokine-induced COX 1 and COX 2 expression by ~60 and 50%, respectively (P < 0.01) (Fig. 12, C and D).

Effect of Curcumin on TNF-α- and IL-1β-Induced Expression of cPLA2, iPLA2, COX 1, and COX 2 in Macrophages

To understand whether curcumin could also block cytokine-mediated stimulation of lipid-metabolizing enzymes in macrophages, we exposed macrophages to the combination of TNF-α and IL-1β while keeping the conditions similar to those for mesangial cells. As seen in Fig. 13, A and B, TNF-α and IL-1β stimulated the expression of cPLA2 and iPLA2, and 10 and 20 μM curcumin dose dependently inhibited the increase in cPLA2 and iPLA2 expression. Figure 13A shows that 10 and 20 μM curcumin decreased the cPLA2 activation by 2.5 (P < 0.01)- and 5-fold (P < 0.001), respectively. iPLA2 expression was blunted 1.5 (P < 0.05)- and 5-fold (P < 0.01) by 10 and 20 μM curcumin, respectively (Fig. 13B). Contrary to mesangial cells, COX 1 expression was not significantly increased in macrophages by the combination of TNF-α and IL-1β, and curcumin did not have any effect on the basal expression of COX 1 (Fig. 13C). The combination of TNF-α and IL-1β increased the expression of COX 2, which was inhibited by 10 and 20 μM curcumin by 64 (P < 0.01) and 85% (P < 0.001), respectively (Fig. 13D).
Fig. 8. Expression of phosphorylated cPLA2, nonphosphorylated cPLA2, and iPLA2 in mesangial cells treated with TNF-α and IL-1β. Mesangial cell were made quiescent by serum starving for 24 h and then treated with 10 ng/ml TNF-α (A), 5 ng/ml IL-1β (B), and a mixture of 10 ng/ml TNF-α + 5 ng/ml IL-1β (C) from 0 to 24 h. During the treatment, the cells were incubated in media with 0.5% serum. Representative Western blots are depicted in the side section of each panel. Values are means ± SD; n = 4–5 experiments. Activation of cPLA2 is depicted as an increase in the phosphorylated cPLA2/nonphosphorylated cPLA2 ratio. The blots were first probed with phosphorylated cPLA2 antibody and then stripped and reprobed with nonphosphorylated cPLA2 antibody. The immunoblots with iPLA2 were first probed with iPLA2 antibody then stripped and normalized to β-actin. Control cells (No TNF-α, No IL-1β, and No cytokine) were kept without any treatment for 24 h.
Fig. 9. Expression of phosphorylated cPLA2, nonphosphorylated cPLA2, and iPLA2 in rat peritoneal macrophage culture treated with TNF-α and IL-1β. Rat peritoneal macrophages were collected 48 h after thioglycolate treatment and plated in 6-well plates. The macrophages were made quiescent by serum starving for 24 h and then treated with 10 ng/ml TNF-α (A), 5 ng/ml IL-1β (B), and a mixture of 10 ng/ml TNF-α+5 ng/ml IL-1β (C) from 0 to 24 h. During treatment, macrophages were incubated in media with 0.5% serum. Representative Western blots are depicted in the side section of each panel. Values are means ± SD; n = 4 experiments in duplicate. Activation of cPLA2 is depicted as an increase in the phosphorylated cPLA2/nonphosphorylated PLA2 ratio. The blots were first probed with phosphorylated cPLA2 antibody and then stripped and reprobed with nonphosphorylated PLA2 antibody. The immunoblots with iPLA2 were first probed with iPLA2 antibody and then stripped and normalized to β-actin. Control cells (No TNF-α, No IL-1β, and No cytokine) were kept without any treatment for 24 h.
DISCUSSION

The uremic environment in CRF is frequently associated with high levels of proinflammatory cytokines, proteinuria, and glomerular and tubulointerstitial damage in both humans and animals (10, 19, 41). Our earlier studies have shown that curcumin can prevent renal failure when it was administered immediately after Nx and before the onset of proteinuria (19); however, in humans early-stage renal failure is frequently not managed per se with treatment commencing at later stages of the disease. Therefore, to better mimic this clinical scenario we initiated curcumin and enalapril therapy 6 wk after Nx when proteinuria was already present. We demonstrated that both curcumin and enalapril significantly reduced proteinuria, glomerulosclerosis, and tubulointerstitial injury, and subsequently renal failure, in association with a downturn in inflammation as mediated by TNF-α and IL-1β. Proinflammatory cytokines

Fig. 10. Expression of COX 1 and COX 2 in mesangial cells treated with TNF-α and IL-1β. Mesangial cells were made quiescent by serum starving for 24 h and then treated with 10 ng/ml TNF-α (A), 5 ng/ml IL-1β (B), and a mixture of 10 ng/ml TNF-α +5 ng/ml IL-1β (C) from 0 to 24 h. During treatment, the cells were incubated in a media with 0.5% serum. Representative Western blots are depicted in the side section of each panel. Values are means ± SD; n = 4–5 experiments. The blots were first probed with either COX 1 or COX 2 antibody and then stripped and normalized to β-actin. Control cells (No TNF-α, No IL-1β and No cytokine) were kept without any treatment for 24 h.
acting on the central nervous system alter appetite and energy metabolism and affect pathways that cause muscle wasting, leading to the cachexia commonly associated with CKD (11). In this study, we observed significant loss of body weight in 16-wk Nx animals, which was abated by both curcumin and enalapril.

Curcumin was as effective as enalapril in preventing the deterioration of renal function, as assessed by biochemical and histological parameters, and did so without a meaningful reduction in BP, unlike enalapril, which substantially reduced BP in these animals. Although curcumin treatment significantly reduced TNF-α and IL-1β levels, it did only for the latter compared with enalapril. ANG II is known to increase TNF-α production (26, 51), and ANG II receptor blockers are known to reduce expression of IL-1β (62). Therefore, we speculated that ANG II might mediate the increase in TNF-α and IL-1β in Nx animals and that curcumin, by blocking ANG II, could abate the increased production of cytokines. This proved not to
be the case in that curcumin had no effect on serum or kidney ANG II concentrations. It can be speculated for enalapril that a timewise reduction in serum and kidney ANG II lessened ANG II-mediated increases in TNF-α and IL-1β. The extent to which serum and tissue ANG II levels declined with enalapril therapy was likely underestimated in these studies since sampling occurred some 24 h after the last dose of enalapril.

Although we are unable as yet to explain the mechanism by which curcumin decreases TNF-α and IL-1β, we explored in these studies the interplay between curcumin and more downstream elements in the inflammatory cascade, PLA2 and COX. Different types of PLA2 have been identified. Broadly, they can be classified into low-molecular-weight secretory PLA2 (sPLA2), high-molecular-weight cPLA2, and iPLA2. Increased sPLA2 activity has been observed in the kidneys of rats with CRF (12), and pharmacological studies using selective inhibitors as well as coexpression experiments have shown that cPLA2 is crucial for the action of sPLA2. In addition, cPLA2 by itself plays a major role in inflammatory diseases and is an attractive therapeutic target in a range of inflammatory disorders (6, 9).

The enzyme cPLA2 is responsible for nearly all of the arachidonic acid release necessary for eicosanoid synthesis, but it also is an essential first step in the synthesis of inflammatory platelet-activating factor (44). cPLA2 hydrolyzes cell membrane phospholipids to produce arachidonic acid and lysophospholipids, playing a key role in the production of inflammatory lipid mediators, mainly eicosanoids. It plays a vital role in the manifestation of inflammation of various systems and organs including the kidney (7, 8). In contrast, iPLA2 shares no specificity toward arachidonic acid-containing phospholipids, and, although less recognized as an inflammatory enzyme, it drives cardiac inflammation in ischemic-reperfusion injury (35, 59). Its activation is a prominent feature in lung injury due to acute inflammation (20). Furthermore, iPLA2 plays a major role in the generation of ROS (61) and monocyte migration (38). Both of these factors are known to provoke inflammation. Curcumin, by blocking iPLA2, can cause decreased monocyte migration, and this can at least in part explain the decreased accumulation of macrophages observed in CRF animals treated with curcumin (19).

Although enalapril did not affect iPLA2 in Nx animals, it and curcumin both significantly reduced the phosphorylated cPLA2/PLA2 ratios in Nx animals. Similar inhibitory effects on PLA2 phosphorylation by curcumin have been observed in cell culture studies (27). ANG II has been shown to increase cPLA2 expression in renal proximal tubules cells (33); therefore, the reduction in cPLA2 by enalapril could be directly related to its
reducing of ANG II generation. In addition, enalapril therapy can reduce ANG II-mediated TNF-α and IL-1β expression, consequently reducing the expression of cPLA2 by these cytokines. TNF-α and IL-1β induce iPLA2 and cPLA2 expression (1, 34, 37), and our in vitro studies demonstrate that TNF-α and IL-1β, given individually, significantly increased iPLA2 and phosphorylated cPLA2/PLA2 expression. When the cytokines were presented together, the increase in both PLA2 isoforms was synergistic and was effectively blocked by curcumin. Therefore, it is possible that curcumin-treated Nx animals had decreased expression of iPLA2 and cPLA2, partly due to the decreased production of TNF-α and IL-1β. In addition, curcumin and enalapril, by reducing arachidonic acid synthesis, decrease the substrate availability for COX 1 and COX 2 enzymes.

Animals with CRF have been shown to have increased expression of COX 1 and COX 2, and blunting of COX expression has been associated with improved renal function in reduction models of renal failure (2, 58). Upregulation of these enzymes can contribute to inflammation, fibrosis/sclerosis, and oxidative stress, associated with renal mass reduction (58). In this study, we observed a significant upregulation of COX 1 and COX 2 in Nx animals, which was abated by curcumin and enalapril treatment. ANG II is also known to increase COX 1 and COX 2 expression (31), and this can explain the significant decrease in both COX isoforms observed in enalapril-treated animals. TNF-α and IL-1β have also been shown to augment COX 2 expression in mesangial cells and macrophages (13, 36, 42). In this study, TNF-α and IL-1β enhanced the expression of COX 2, and the outcome was synergistic when the cytokines were given together. The effects of the cytokines were mostly similar in both cell types studied. Curcumin significantly reduced cytokine-manifested expression of COX 2, albeit only at the highest concentration in mesangial cells and macrophages. As seen with PLA2 expression, the cytokine mixtures significantly increased COX 2 expression in the macrophages at 10 and 24 h. However, unlike their effect on PLA2, the increase in expression at an earlier time point (10 h) was not observed in mesangial cells.

Although COX 1 is relatively resistant to stimulation by any mediator, high concentrations (25 ng/ml) of TNF-α and IL-1β have been shown to induce COX 1 expression in mesangial cells (55). In the present study, increased expression of COX 1 was not observed when TNF-α or IL-1β was given independently; however, the combination elicited augmented expression of COX 1 at 24 h only in mesangial cells, which was blocked by curcumin. Studies to determine the selectivity of curcumin-mediated inhibition of COX 1 and COX 2 have shown that curcumin and its metabolites had significantly higher inhibitory effects on the peroxidase activity of COX 1 than on COX 2 (27). It is to be cautioned that COX-derived prostanoids play an important role in maintaining renal func-
tion, body fluid homeostasis, and blood pressure, particularly in volume-contracted states (23, 24). COX 2 inhibitors lead to sodium and volume retention and may predispose to or exacerbate hypertension; however, there is also increasing evidence that COX 2 expression increases in progressive renal injury (24). Furthermore, PGE₂ is the major renal COX-derived metabolite in the kidney and acts on EP receptors (PGE₂ receptors) to influence renal vascular resistance. Although PGE₂ has been demonstrated to increase renal blood flow and glomerular filtration rate, EP₁ and EP₃ receptor activation can result in constriction of the renal vasculature (29).

In summary, curcumin has been proven to be as effective as enalapril in forestalling renal failure progression in Nx animals even though it did not demonstrate the same vasodepressor response as enalapril. It is tempting to propose that curcumin and enalapril treatment not only blocked but reversed progression of the disease. However, the data on segmental sclerosis and other biochemical parameters such as creatinine and BUN (6-wk Nx animals vs. treated animals) indicate that the treatments did not reverse the disease process but attenuated the progression. Although not a conventional PLA₂ or a COX inhibitor, the action of enalapril on these enzymes is probably a function of its reducing of ANG II concentrations and lends itself to what is a growing body of information on pleiotropic effects of angiotensin-converting enzyme inhibitors; however, the role of blood pressure reduction in any of these processes is unclear at this time.

Curcumin is a unique compound with varied pharmacological properties. It is known to favorably affect proinflammatory cytokines, adhesion molecules, TGF-β, PPARγ, NF-κB, and is also a potent antioxidant (4, 19). Either individually or in sum, these pharmacological features can have a positive impact in the setting of renal failure. Tetrahydrocurcumin, and other metabolites of curcumin, which inhibit NF-κB, COX 1, and 5-lipoxygenase, although less effectively than the parent, may have contributed to the observed findings (27, 30, 49).

The uremic milieu is characterized by an increased secretion of proinflammatory cytokines, including TNF-α and IL-1β (54), and although we could not address the mechanism by which curcumin blocks TNF-α and IL-1β, we speculate that by lessening the progression rate of renal failure (as evidenced by decreased BUN and creatinine) curcumin removed the stimulus to an increased secretion of these cytokines. In this study, we have shown that curcumin by blocking TNF-α and IL-1β prevents the upregulation of cPLA₂, iPLA₂, and COX isoforms, which might otherwise contribute to the deterioration of renal function in Nx animals.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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