

Hypercalcemia stimulates expression of intrarenal phospholipase A2 and prostaglandin H synthase-2 in rats. Role of angiotensin II AT1 receptors.

H Mangat, ... , L N Peterson, K D Burns

J Clin Invest. 1997;100(8):1941-1950. <https://doi.org/10.1172/JCI119725>.

Research Article

In chronic hypercalcemia, inhibition of thick ascending limb sodium chloride reabsorption is mediated by elevated intrarenal PGE₂. The mechanisms and source of elevated PGE₂ in hypercalcemia are not known. We determined the effect of hypercalcemia on intrarenal expression of cytosolic phospholipase A₂ (cPLA₂), prostaglandin H synthase-1 (PGHS-1), and prostaglandin H synthase-2 (PGHS-2), enzymes important in prostaglandin production. In rats fed dihydrotachysterol to induce hypercalcemia, Western blot analysis revealed significant upregulation of both cPLA₂ and PGHS-2 in the kidney cortex and the inner and outer medulla. Immunofluorescence localized intrarenal cPLA₂ and PGHS-2 to interstitial cells of the inner and outer medulla, and to macula densa and cortical thick ascending limbs in both control and hypercalcemic rats. Hypercalcemia had no effect on intrarenal expression of PGHS-1. To determine if AT₁ angiotensin II receptor activation was involved in the stimulation of cPLA₂ and PGHS-2 in hypercalcemia, we treated rats with the AT₁ receptor antagonist, losartan. Losartan abolished the polydipsia associated with hypercalcemia, prevented the increase in cPLA₂ protein in all regions of the kidney, and diminished PGHS-2 expression in the inner medulla. In addition, losartan completely prevented the increase in urinary PGE₂ excretion in hypercalcemic rats. Intrarenal levels of angiotensin II were unchanged in hypercalcemia. These data indicate that hypercalcemia stimulates intrarenal cPLA₂ and PGHS-2 protein expression. Our results further support a role [...]

Find the latest version:

<https://jci.me/119725/pdf>



Hypercalcemia Stimulates Expression of Intrarenal Phospholipase A₂ and Prostaglandin H Synthase-2 in Rats

Role of Angiotensin II AT₁ Receptors

Harman Mangat,* Linda N. Peterson,** and Kevin D. Burns*§

*Department of Physiology, †Department of Pediatrics, and §Department of Medicine, University of Ottawa and Ottawa General Hospital, Ottawa, Ontario, Canada K1H 8M5

Abstract

In chronic hypercalcemia, inhibition of thick ascending limb sodium chloride reabsorption is mediated by elevated intrarenal PGE₂. The mechanisms and source of elevated PGE₂ in hypercalcemia are not known. We determined the effect of hypercalcemia on intrarenal expression of cytosolic phospholipase A₂ (cPLA₂), prostaglandin H synthase-1 (PGHS-1), and prostaglandin H synthase-2 (PGHS-2), enzymes important in prostaglandin production. In rats fed dihydrotachysterol to induce hypercalcemia, Western blot analysis revealed significant upregulation of both cPLA₂ and PGHS-2 in the kidney cortex and the inner and outer medulla. Immunofluorescence localized intrarenal cPLA₂ and PGHS-2 to interstitial cells of the inner and outer medulla, and to macula densa and cortical thick ascending limbs in both control and hypercalcemic rats. Hypercalcemia had no effect on intrarenal expression of PGHS-1. To determine if AT₁ angiotensin II receptor activation was involved in the stimulation of cPLA₂ and PGHS-2 in hypercalcemia, we treated rats with the AT₁ receptor antagonist, losartan. Losartan abolished the polydipsia associated with hypercalcemia, prevented the increase in cPLA₂ protein in all regions of the kidney, and diminished PGHS-2 expression in the inner medulla. In addition, losartan completely prevented the increase in urinary PGE₂ excretion in hypercalcemic rats. Intrarenal levels of angiotensin II were unchanged in hypercalcemia. These data indicate that hypercalcemia stimulates intrarenal cPLA₂ and PGHS-2 protein expression. Our results further support a role for angiotensin II, acting on AT₁ receptors, in mediating this stimulation. (*J. Clin. Invest.* 1997. 100:1941–1950.) **Key words:** prostaglandin H synthase-1 • prostaglandin E₂ • losartan • kidney • Western blot

Introduction

The association of hypercalcemia with disturbances in renal concentrating ability, water intake, and loop of Henle function has been widely recognized in both humans (1, 2) and experimental animals (3, 4). Studies in rats show that chronic hypercalcemia is associated with inhibition of thick ascending limb NaCl reabsorption (5). Renal PGE₂ excretion is strikingly elevated in hypercalcemic rats (6, 7), suggesting that PGE₂ may be involved in mediating this defective NaCl reabsorption. In rats with vitamin D-induced chronic hypercalcemia, outer medullary PGE₂ levels are increased, and PGE₂ appears to mediate the inhibition of thick ascending limb NaCl transport at a step proximal to the generation of cAMP (8).

Although differences exist between animal species, PGE₂ is the predominant (80–90% of the measured prostaglandins) renal prostaglandin in all species. The proximal and distal convoluted tubules synthesize very small amounts of PGE₂, while the thin descending limb of Henle, the medullary thick ascending limb of Henle, and the medullary and cortical collecting tubules possess greater synthetic capacity (9, 10). Medullary interstitial cells are also able to produce abundant amounts of PGE₂ (11).

The availability of arachidonic acid is a rate-limiting step controlling the synthesis of prostaglandins. Release of arachidonic acid from membrane phospholipids is regulated primarily by phospholipase A₂, an enzyme that catalyzes hydrolysis of the ester linkage at the *sn*-2 position of membrane phospholipids to produce free fatty acids and lysophospholipids. A high molecular mass (85-kD) cytosolic phospholipase A₂ (cPLA₂)¹ has been identified in numerous tissues, including rat kidney (12). Prostaglandin H synthase (PGHS) is another key enzyme involved in prostaglandin synthesis. PGHS exhibits both bisoxxygenase (cyclooxygenase) activity catalyzing prostaglandin G₂ formation from arachidonic acid, and peroxidase activity, which reduces the 15-hydroperoxyl group of prostaglandin G₂ to the 15-OH of prostaglandin H₂. Mammalian cells contain two isoforms of PGHS (PGHS-1 and PGHS-2), which share ~60% amino acid identity within the same species (13).

PGHS-1 mediates basal production of PGE₂ (14), and within the mouse kidney is constitutively expressed in arteries and arterioles, glomeruli, collecting ducts, and medullary interstitial cells, with no immunoreactivity detected in proximal or distal convoluted tubules, loop of Henle, or macula densa (15, 16). PGHS-2 has been termed the inducible form of the enzyme since its expression is often undetectable unless induced by mitogenic or proinflammatory agents (17–21). In the kidney, however, Harris et al. detected PGHS-2 immunoreactivity in

A portion of this work was presented in abstract form (1995. *J. Am. Soc. Nephrol.* 6[3]: 540a).

Address correspondence to Dr. Kevin D. Burns, Division of Nephrology, Ottawa General Hospital, 501 Smyth Road, Rm. N-8, Ottawa, Ontario, Canada K1H 8L6. Phone: 613-737-8919; FAX: 613-737-8141; E-mail: kburns@ogh.on.ca

Received for publication 14 October 1996 and accepted in revised form 11 August 1997.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/97/10/1941/10 \$2.00

Volume 100, Number 8, October 1997, 1941–1950

<http://www.jci.org>

1. *Abbreviations used in this paper:* Ang II, angiotensin II; cPLA₂, cytosolic phospholipase A₂; DHT, dihydrotachysterol; PGHS, prostaglandin H synthase.

the absence of inflammation, in the macula densa, cortical thick ascending limb, and interstitial cells of the inner medulla, with no immunoreactivity in arterioles, glomeruli, or collecting ducts (15).

The source and mechanisms for elevated renal PGE₂ levels in hypercalcemia are unknown. In this study, we used Western blot analysis and immunofluorescence to determine the effect of hypercalcemia on protein expression of cPLA₂, PGHS-1, and PGHS-2 in the rat kidney. Our results reveal that hypercalcemia stimulates expression of cPLA₂ and PGHS-2 in kidney cortex, and in inner and outer medulla. Furthermore, our data suggest a key role for activation of the AT₁ angiotensin II (Ang II) receptor in mediating hypercalcemia-induced stimulation of cPLA₂ and PGHS-2 expression.

Methods

Animals. Male Sprague-Dawley rats (250–320 g) were used for this study. Hypercalcemia was induced by feeding the rats a normal electrolyte diet containing 4.25 mg/kg diet of dihydrotachysterol (DHT) for 1–5 d, as previously described (5). Control measurements were taken from rats pair-fed (7 g/100 g body wt./d) the identical electrolyte diet without DHT. All animals were allowed free access to water. In some studies, hypercalcemic and control rats were given losartan (15 mg/kg/d) (22) in the drinking water. At a predetermined time during the study period, rats were anesthetized with Somnitol (60 mg/kg i.p.), and cardiac puncture was performed to obtain blood for measurement of ionized calcium and pH by ion-specific electrodes (Stat Analyzer™ model 634; Ciba Corning, Medfield, MA). Reported blood calcium was corrected to pH 7.40.

Western blot analysis. One kidney was rapidly removed and immediately placed in 4°C homogenization buffer (50 mM Hepes [pH 7.4], 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10% vol/vol glycerol). The kidneys were decapsulated and cut into 1-mm slices. The inner medulla, outer medulla, and cortex were dissected from each slice, separated, placed into fresh buffer, and homogenized for 30 s using a polytron. Tissue homogenates were centrifuged at 10,000 g for 5 min, and protein concentrations of the supernatants were measured using the Bradford assay (23) with BSA as standard. Total protein equivalents for each lysate (5-µg inner medulla; 10-µg outer medulla, and 20-µg cortex) were heated at 100°C for 5 min in sample buffer containing 63 mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 2% mercaptoethanol, and 1 mg/ml of bromophenol blue. The samples were separated on 7.5% SDS/polyacrylamide minigels using the Mini PROTEAN II electrophoretic apparatus, and transferred to nitrocellulose membranes using the Mini Trans blot electrophoretic transfer system (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Membranes were blocked overnight at room temperature with a solution of 5% fat-free dried milk in Tris-buffered saline–0.1% Tween-20 (TBS-T). Separate membranes were incubated at room temperature with either a polyclonal PGHS-1 rabbit IgG (1:1000 dilution) for 18 h, a rabbit anti-murine polyclonal antibody to PGHS-2 (1:1000 dilution) for 18 h, or with polyclonal cPLA₂ antiserum for 1 h (1:2000 dilution). The membranes were washed with TBS-T and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution) for 30 min at room temperature. Immunoreactive proteins were visualized on x-ray film, using the enhanced chemiluminescence method.

Immunohistochemistry. Kidneys were removed, immediately fixed in 10% formalin, embedded in paraffin, and sections (4-µm) were mounted on glass slides. For immunostaining, the sections were permeabilized with SDS followed by incubation with 1% BSA to minimize nonspecific antibody binding. The sections were then incubated for 24 h at 4°C with either the polyclonal cPLA₂ antiserum (1:100 dilution) or the rabbit anti-murine antiserum (1:50 dilution) for PGHS-2 immunolocalization. All sections were also incubated with a polyclonal rabbit anti-mouse antibody to bNOS (1:100 dilution), known

to localize to the macula densa (24). Primary antibody binding for PGHS-2 or cPLA₂ was visualized with FITC-conjugated goat anti-rabbit IgG (1:50 dilution). Goat anti-mouse IgG conjugated to Texas red (1:100 dilution) was used for the detection of bNOS protein. All slides were mounted in glycerol containing *p*-phenylenediamine to inhibit fading. Sections were viewed and photographed using an Axioptan Universal™ microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a fluorescence illuminator and an HBO 50 mercury lamp (Carl Zeiss, Inc.).

Determination of urinary PGE₂ by radioimmunoassay. Urine was collected over a 24-h period from rats fed the control or DHT-containing diet for 3 or 5 d. An aliquot of urine was acidified to pH 4.0 and centrifuged at 1,200 g for 30 min. The supernatant was extracted with ethyl acetate/methanol using Bond Elut C18 columns (Varian Associates, Inc., Palo Alto, CA). In preliminary experiments, 94% of [³H]PGE₂ added to the supernatant was recovered from the C18 column. The ethyl acetate/methanol extract was dried at room temperature under a stream of nitrogen, reconstituted in assay buffer, and radioimmunoassay was performed according to the procedure outlined in the assay kit. Results for urinary PGE₂ are expressed as nanograms of PGE₂ excreted over a 24-h period.

Measurement of intrarenal Ang II. Rats were fed the control or DHT-containing diet for 3 d, and intrarenal levels of Ang II were measured by HPLC and radioimmunoassay using the method of Ruzicka et al. (25). In brief, kidneys were perfused in situ via intracardiac injection with PBS (pH 7.4) to remove blood, and were then removed and snap-frozen in liquid nitrogen. Snap-frozen kidneys were weighed, minced, and then boiled for 20 min in 1 M acetic acid. Tissue was then homogenized for 1 min using a hand-held homogenizer (Tissue Tearor™; Biospec Products Inc., Racine, WI), and centrifuged at 10,000 g for 10 min at 4°C. The aqueous phase was removed, passed through a C18 Sep-Pak cartridge (Waters, Milford, MA), and eluted with 4% acetic acid in ethanol. Eluants were dried in a Speed Vac concentrator for further analysis. HPLC separation of Ang II was kindly performed by the laboratory of Dr. F. Leenen (University of Ottawa), according to their published protocol (25). After HPLC, Ang II levels were measured in the eluants by radioimmunoassay, using [¹²⁵I]Ang II and rabbit antiserum to Ang II. Standard curves were prepared using Asn¹-Val⁵-Ang II as substrate. Results are expressed as fmol Ang II/g kidney.

Densitometric and statistical analyses. The intensity of bands obtained from Western blot analysis was quantified by scanning densitometry using a software image analysis program (Image 1.47). Protein bands from hypercalcemic rats were compared to the intensity of proteins from pair-fed control rats. An arbitrary density of one was assigned to the band from the control rat kidney. Preliminary experiments revealed that band intensity on autoradiographs for cPLA₂, PGHS-1, and PGHS-2 increased linearly with quantity of protein loaded (not shown).

Data are expressed as means ± SE. Significance was assigned at *P* < 0.05. Since protein bands from hypercalcemic rats were normalized to bands from control rats, the data do not follow a normal distribution. Therefore, statistical analysis of the Western blots was performed using the nonparametric Kruskal-Wallis test in combination with the Dunn's multiple comparison post-hoc test to examine the differences between individual group means. All other data were analyzed using a one-way ANOVA followed by the Tukey post-hoc test.

Materials. Normal electrolyte diet with and without DHT was obtained from ICN Nutritional Biochemicals (Cleveland, OH); losartan was kindly donated by Merck & Co., Inc. (Rahway, NJ); BSA, Mini PROTEAN II electrophoretic apparatus nitrocellulose membranes, and the Mini Trans blot electrophoretic transfer system were from Bio-Rad Laboratories (Mississauga, Ontario, Canada); goat anti-rabbit IgG conjugated to horseradish peroxidase and enhanced chemiluminescence kit were from Amersham Corp. (Oakville, Ontario, Canada); PGHS-1 rabbit IgG was from Oxford Biomedical Research Inc. (Oxford, MI); PGHS-2 rabbit IgG was from Cayman Chemical Co., Inc. (Ann Arbor, MI); cPLA₂ antiserum was kindly donated by The

Table I. Ionized Calcium Levels in Rats Fed DHT-containing Diet for 0 to 5 d

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Ionized calcium (mmol/liter)	1.33±0.03 (5)	1.39±0.01 (5)	1.52±0.01* (4)	1.65±0.05‡ (4)	1.69±0.04‡ (5)	1.69±0.02‡ (5)

Values are means±SE. Numbers in parentheses represent number of rats. **P* < 0.01 vs Day 0, ‡*P* < 0.001 vs. Day 0.

Genetics Institute (Cambridge, MA); FITC-conjugated goat anti-rabbit IgG was from Vector Laboratories, Inc. (Burlingame, CA); anti-bNOS antibody and goat anti-mouse IgG conjugated to Texas red were from Bio/Can (Mississauga, Ontario, Canada); PGE₂ radioimmunoassay kit was from DuPont-NEN (Boston, MA); Bond Elut C18 columns were from Varian Associates Inc. (Palo Alto, CA); Ang II antiserum was kindly provided by Dr. F. Leenen, University of Ottawa (Ottawa, Ontario, Canada); C-18 Sep-Pak cartridges were from Waters (Millford, MA); and Asn¹-Val⁵-Ang II was from Sigma Chemical Co. (St. Louis, MO).

Results

Hypercalcemia was induced by feeding rats a DHT-containing diet. Blood-ionized calcium concentration, presented in Table I, was significantly elevated after 2 d of diet (control, 1.33±0.03

mmol/liter vs. DHT, 1.52±0.01 mmol/liter; *P* < 0.01, *n* = 4) and increased to a maximum on day 3 (1.65±0.05 mmol/liter; *P* < 0.001 vs. day 0; *n* = 4). Levels remained significantly elevated in DHT-fed rats for the remainder of the study period (1.69±0.02 mmol/liter, day 5; *P* < 0.001 vs. day 0; *n* = 5). To determine whether intrarenal expression of PGHS-1, PGHS-2, and cPLA₂ is affected by hypercalcemia, Western blots were performed on homogenates of inner medulla, outer medulla, and cortex from rats fed the DHT-containing diet for up to 5 d.

PGHS-1. PGHS-1 protein was detected on Western blots as a single band of ~ 75 kD in kidneys from both control and hypercalcemic rats. There was no significant change in expression of this protein with hypercalcemia over the period studied in any of the kidney regions (Fig. 1).

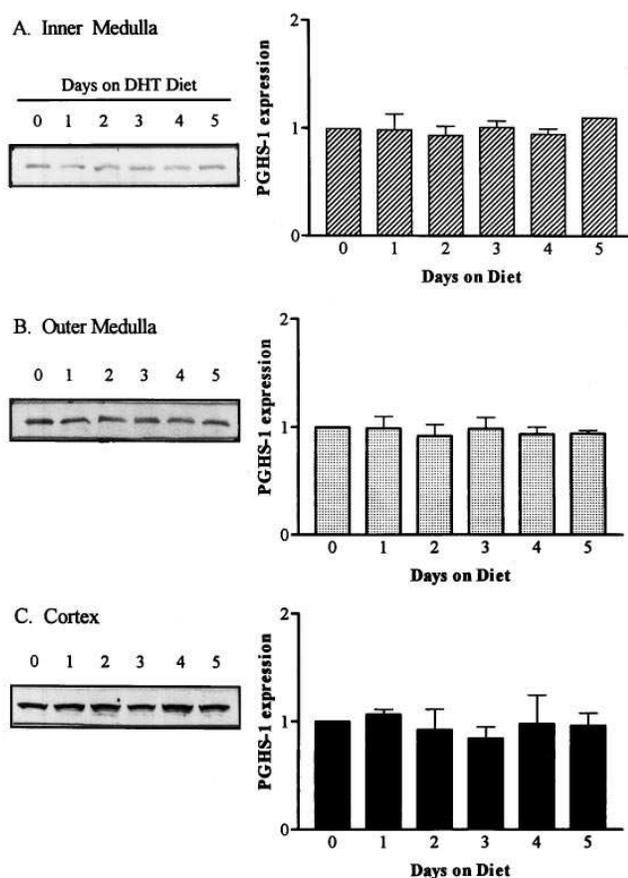


Figure 1. Intrarenal PGHS-1 expression in hypercalcemia. Representative Western blots (left) and densitometric analyses (right) demonstrating levels of PGHS-1 protein in the (A) inner medulla (5 µg protein/well), (B) outer medulla (10 µg protein/well), and (C) cortex (20 µg protein/well) of rats fed DHT-containing diet for 0 to 5 d. Densitometric values represent the means±SE of three experiments.

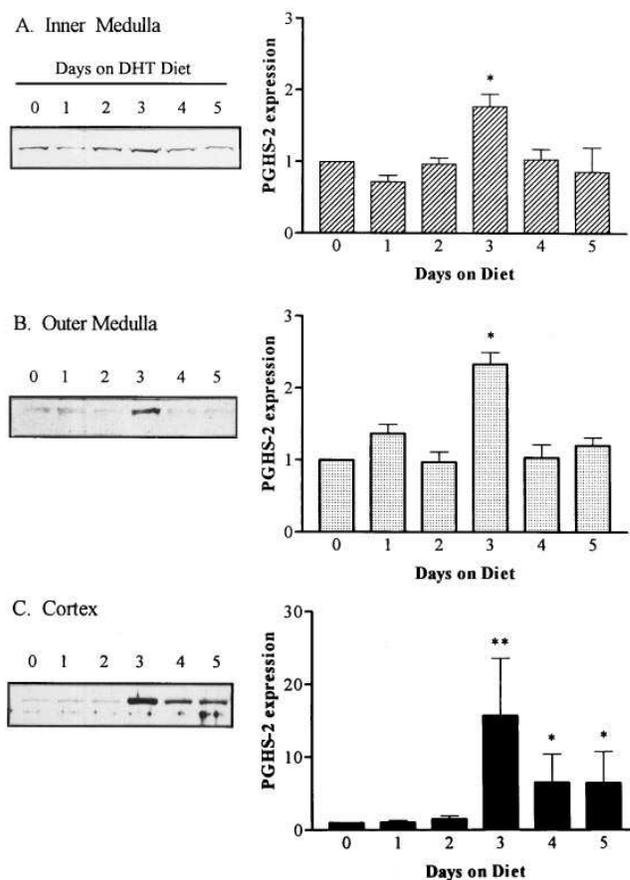


Figure 2. Time course for expression of PGHS-2 in hypercalcemia. PGHS-2 expression was assessed by Western blots (left) and densitometric analyses (right) in the (A) inner medulla (5 µg protein/well), (B) outer medulla (10 µg protein/well), and (C) cortex (20 µg protein/well) of rats fed DHT-containing diet for 0 to 5 d. Densitometric values represent the means±SE of three to six experiments. **P* < 0.05 vs. day 0, ***P* < 0.01 vs. day 0.

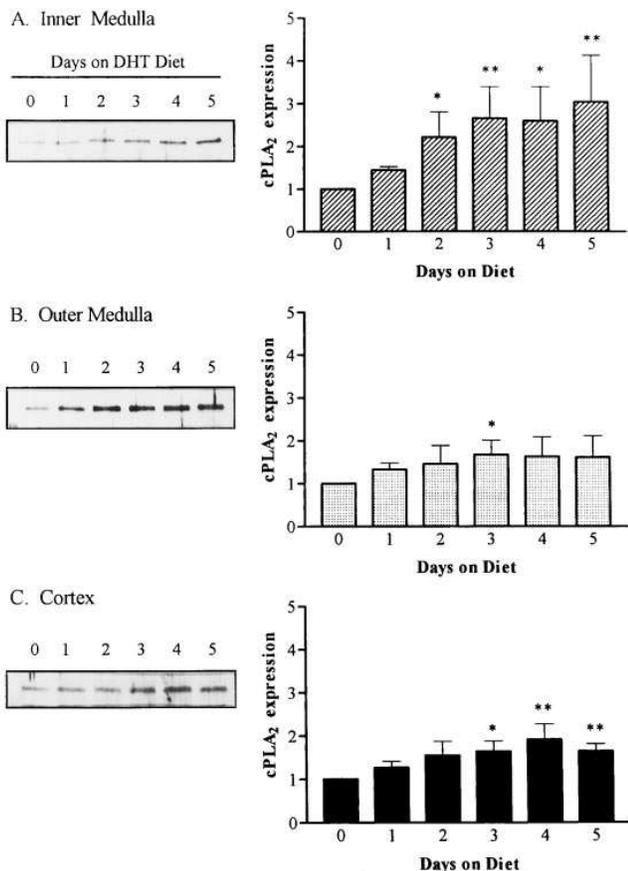


Figure 3. Expression of cPLA₂ in hypercalcemic rats. Representative Western blots (left) and densitometric analyses (right) demonstrating protein expression in the (A) inner medulla (5 μ g protein/well), (B) outer medulla (10 μ g protein/well), and (C) cortex (20 μ g protein/well) of rats fed DHT-containing diet for 0 to 5 d. Densitometric values represent the means \pm SE of 4–6 experiments. * P < 0.05 vs. day 0, ** P < 0.01 vs. day 0.

PGHS-2. PGHS-2 was expressed in kidneys from both control and hypercalcemic rats, and appeared as a single band on most blots at \sim 72 kD. PGHS-2 protein expression in both inner and outer medulla was significantly increased after 3 d on the DHT diet (fold increase vs. control: inner medulla, 1.8 ± 0.2 ; P < 0.05, n = 3; outer medulla, 2.3 ± 0.2 ; P < 0.05, n = 3), after which expression returned to control levels (Fig. 2, A and B). PGHS-2 expression in the cortex (Fig. 2 C) was also increased on day 3 (fold increase vs. control: 15.7 ± 7.9 ; P < 0.01, n = 6) and remained significantly elevated up to day 5.

cPLA₂. Using a specific antiserum for cPLA₂, an immunoreactive protein of \sim 110 kD was observed (Fig. 3). In the inner medulla, outer medulla, and cortex, there were significant increases in cPLA₂ protein expression at 3 d on the DHT diet (fold increase vs. control: inner medulla, 2.7 ± 0.7 ; P < 0.01, n = 4; outer medulla, 1.5 ± 0.3 ; P < 0.05, n = 6; cortex, 1.6 ± 0.2 ; P < 0.05, n = 6). Levels of cPLA₂ protein in the outer medulla at days 4 and 5 of DHT diet were not statistically different from control values. cPLA₂ protein expression in the inner medulla and cortex, however, remained significantly increased at days 4 and 5 of the study.

Immunohistochemistry. To determine which cells might mediate the changes in expression of PGHS-2 and cPLA₂ in

hypercalcemia, immunofluorescence studies were performed. Intrarenal localization of PGHS-2 and cPLA₂ by immunohistochemistry did not differ between control and hypercalcemic rats. PGHS-2 immunoreactivity in the inner and outer medulla was localized primarily to interstitial cells (Fig. 4, A and B). In the cortex, intense staining for PGHS-2 was observed in the macula densa region, as identified by colocalization with bNOS (24), and in the adjoining cortical thick ascending limb (Fig. 4, C, D, and E).

In the inner medulla, cPLA₂ immunoreactivity was detected mainly in the interstitial cells with a faint signal in epithelial cells of the inner medullary collecting ducts (Fig. 5 A). The interstitial cells of the outer medulla also showed positive immunoreactivity for cPLA₂ (Fig. 5 B). In the cortex, cPLA₂ immunoreactivity was detected in the macula densa region (Fig. 5, C, D, and E).

Role of AT₁ Ang II receptors. Ang II has been reported to increase prostaglandin production in a number of systems (26–28). In our previous studies, circulating plasma renin levels did not increase significantly in hypercalcemic rats with access to water, although pair-water feeding was associated with significantly higher levels (5). Studies were performed to investigate the effect of hypercalcemia on intrarenal levels of Ang II, determined by HPLC and radioimmunoassay. After 3 d of hypercalcemia, a time point at which we observed increased expression of cPLA₂ and PGHS-2, levels of intrarenal Ang II did not significantly differ from control values (Fig. 6). These values were comparable to those previously reported (29).

To determine whether AT₁ Ang II receptor activation plays a role in the regulation of cPLA₂ and PGHS-2 in hypercalcemia, losartan, a specific AT₁ Ang II receptor antagonist, was administered in the drinking water of rats on control and DHT diets for 3 d, a time at which these proteins were significantly elevated in hypercalcemia (Figs. 2 and 3). Losartan had no effect on serum calcium in hypercalcemic rats (hypercalcemic, 1.62 ± 0.05 mmol/liter vs. hypercalcemic + losartan, 1.61 ± 0.06 mmol/liter; P = NS, n = 5). Water intake of all animals was measured daily, and the dose of losartan was adjusted to ensure that 15 mg/kg/d was being administered. Water intake was significantly increased in hypercalcemic animals (control, 19.6 ± 1.4 ml/100 g body wt/d vs. hypercalcemic, 27.4 ± 2.3 ml/100 g body wt/d; P < 0.05, n = 5) in agreement with previous studies (5, 6). Treatment of hypercalcemic animals with losartan, however, significantly reduced water intake (hypercalcemic + losartan, 20.4 ± 1.1 ml/100 g body wt/d; P = NS vs. control, n = 11) (Fig. 7).

As shown in Fig. 8, in rats on control diet, cPLA₂ protein expression was not affected by losartan. In hypercalcemic rats, however, losartan treatment significantly inhibited the increase in cPLA₂ protein in the inner medulla, outer medulla, and cortex. Losartan also abolished the increase in PGHS-2 expression in the inner medulla of hypercalcemic rats, but had no effect on increased expression of PGHS-2 in the cortex and outer medulla (Fig. 9).

Urinary excretion of PGE₂ between days 2 and day 3 did not differ significantly between control and hypercalcemic rats (control, 417 ± 116 ng/d vs. hypercalcemic, 535 ± 193 ng/d; P = NS, n = 4). In contrast, and in agreement with previous reports (6, 7), urinary PGE₂ excretion measured at 5 d was significantly elevated in hypercalcemic rats when compared with controls (control, 347 ± 22 ng/d vs. hypercalcemic, 1706 ± 210 ng/d; P < 0.001, n = 3) (Fig. 10). In hypercalcemic rats treated

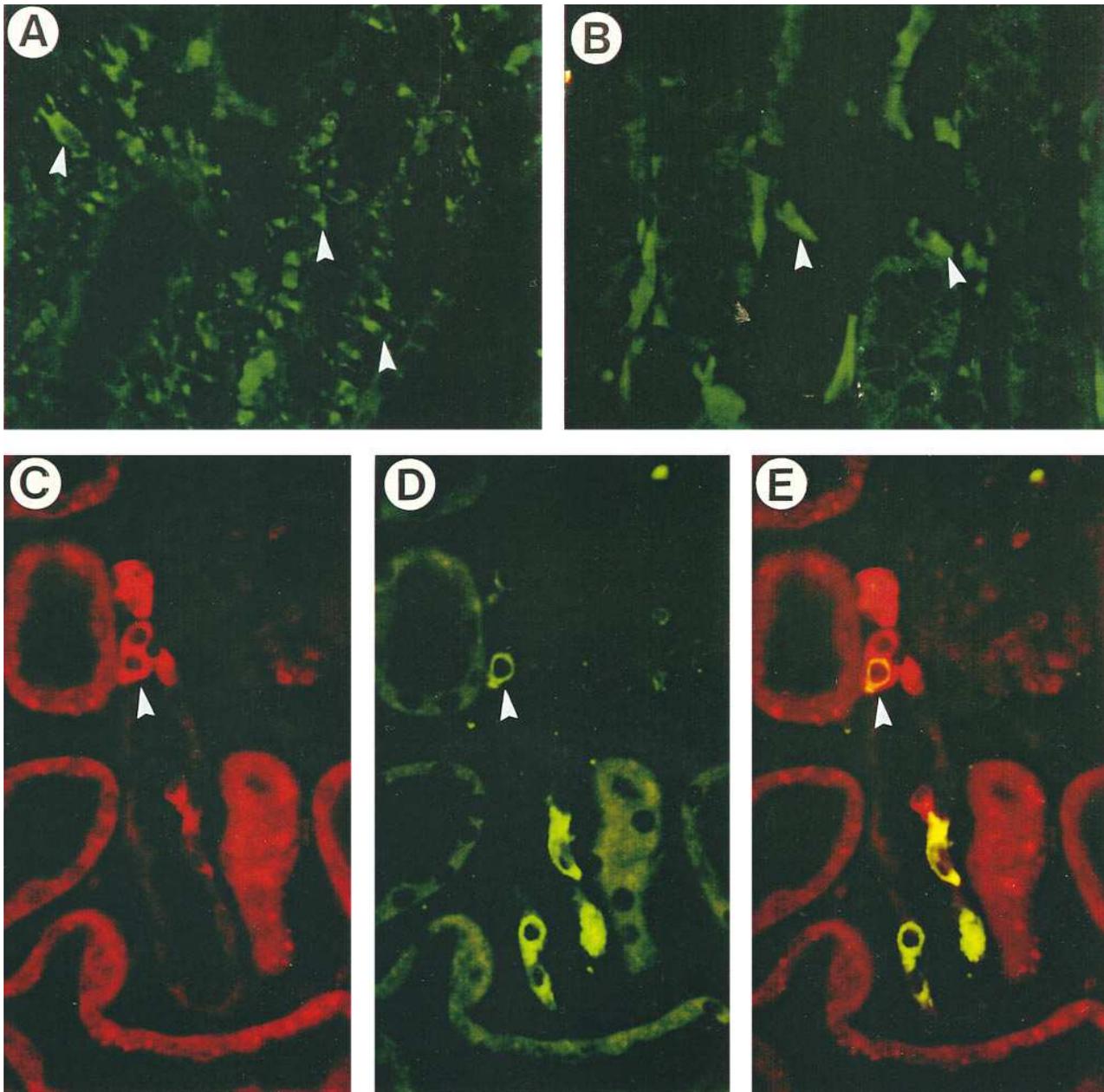


Figure 4. PGHS-2 and bNOS immunostaining in kidney tissue from hypercalcemic rats. (A) Inner medulla, demonstrating PGHS-2 immunofluorescence in interstitial cells (*arrowheads*), (B) PGHS-2 immunofluorescence demonstrating staining of interstitial cells in outer medulla (*arrowheads*), (C) bNOS staining of macula densa in the cortex (*arrowhead*), (D) PGHS-2 immunofluorescence of the same field as in (C) showing staining of macula densa, identified by clustering of nuclei and cortical thick ascending limb (*arrowhead*), and (E) colocalization of bNOS and PGHS-2 demonstrating staining in macula densa (*arrowhead*). Immunostaining shown here is representative of four experiments. Identical localization was observed in all three regions of control rats (not shown). $\times 360$.

with losartan, however, the increase in urinary PGE_2 was completely prevented (hypercalcemic + losartan, 705 ± 119 ng/d; $P = NS$ vs. control, $n = 4$).

Discussion

Inhibition of thick ascending limb chloride reabsorption in hypercalcemia has been linked to increased prostaglandin levels in the kidney (8). There have been no studies, however, ad-

ressing either regulation of enzymes involved in prostaglandin synthesis or determination of the source of increased prostaglandins in hypercalcemia. The results from this study demonstrate that hypercalcemia causes upregulation of intrarenal $cPLA_2$ and PGHS-2, the two major enzymes involved in prostaglandin synthesis, without altering intrarenal localization, as determined by immunofluorescence. Furthermore, we have demonstrated a role for Ang II, acting on AT_1 receptors, in mediating this upregulation, and in increasing urinary PGE_2 excretion in hypercalcemia.

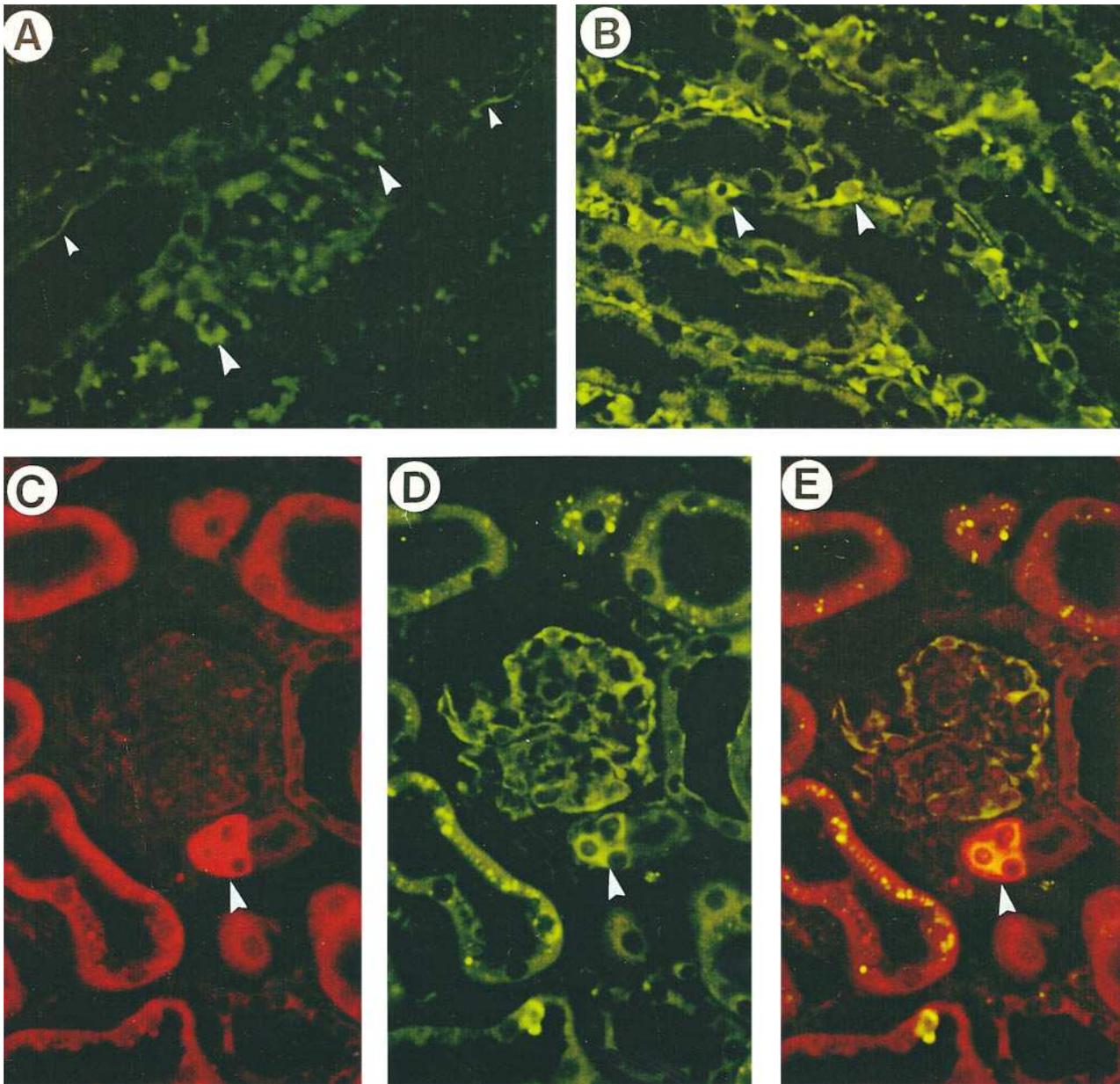


Figure 5. Immunostaining of cPLA₂ and bNOS protein in hypercalcemia. (A) Inner medulla, demonstrating cPLA₂ immunofluorescence mainly in interstitial cells (*large arrowheads*) with a faint signal in inner medullary collecting ducts (*small arrowheads*), (B) cPLA₂ immunofluorescence demonstrating staining of interstitial cells in outer medulla (*arrowheads*), (C) bNOS staining of macula densa in the cortex (*arrowhead*), (D) cPLA₂ immunofluorescence in the cortex, and (E) immunofluorescence of the same field as in (C) and (D) demonstrating colocalization of cPLA₂ and bNOS to the macula densa in the cortex (*arrowhead*). Immunostaining shown here is representative of four experiments. Similar results were observed in control rats (not shown). $\times 360$.

In DHT-induced hypercalcemia, blood calcium levels are significantly elevated by 2 d, as is water intake, two events that are independent of, and not responsible for, the concentrating defect that is evident by day 3 (6). The concentrating defect has been shown to be mediated by intrarenal PGE₂, levels that are significantly elevated in hypercalcemia (8). A possible mechanism for this defect is suggested by the recent study of Kaji et al. (30), who demonstrated that PGE₂ inhibits Na-K-2Cl cotransport in medullary thick ascending limb cells by down-regulating the number of functioning cotransporters.

The rate-limiting step in prostaglandin synthesis is the PLA₂-mediated release of arachidonic acid from membrane phospholipids (31). cPLA₂ has been isolated and purified from kidney tissue (12), and its activity has been studied in isolated glomerular mesangial cells (32). In vivo regulation of renal cPLA₂ and its specific cellular localization in the kidney, however, remains unclear. In this work, expression of cPLA₂ was evident in normal rat kidney tissue, and we demonstrated significant upregulation after 3 d of DHT treatment, suggesting increased endogenous availability of arachidonic acid in the

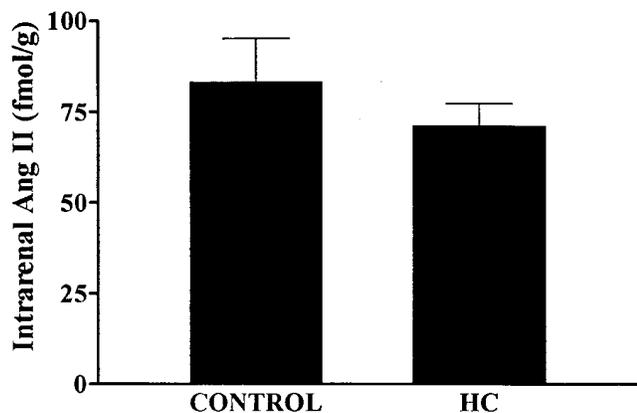


Figure 6. Intrarenal Ang II levels in control and hypercalcemic rats. Rats were fed control or DHT-containing (*HC*) diet for 3 d. Ang II levels were measured by HPLC and radioimmunoassay. Data are expressed as means \pm SE ($n = 4$).

kidneys of these animals. In addition, we localized immunoreactive cPLA₂, mainly to interstitial cells of the inner and outer medulla (which are well known to synthesize and release prostaglandins [33, 34]) and to the macula densa.

Induction of PGHS-2, the other critical enzyme involved in prostaglandin synthesis, is associated with inflammation, mitogenesis, and ovulation (13, 35). Expression of PGHS-2 mRNA in vitro parallels that of *c-fos*, leading to the classification of PGHS-2 as an immediate early response gene (36). Rapid transient activation of PGHS-2, and subsequent return to baseline levels, has been observed in a variety of cultured cell systems (18, 37) including kidney mesangial cells (34). To date, however, renal expression and regulation of this enzyme in vivo has not been fully elucidated. PGHS-2 is expressed under unstimulated conditions in the kidney (15); our work provides further support for this finding. In addition, we demonstrate that the expression pattern of PGHS-2 in this in vivo model is consistent with observations in cultured cells. To this end, we

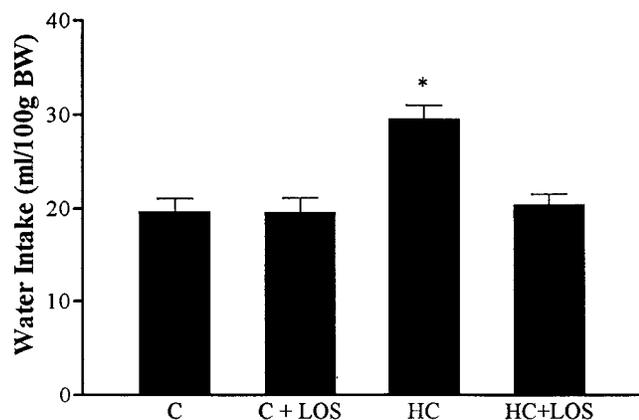


Figure 7. Effect of losartan on water intake. Water intake over 24 h was measured in rats fed the control diet (*C*) alone, the control diet with losartan in the drinking water (*C + LOS*), the DHT-containing diet (*HC*), and the DHT-containing diet and losartan in the drinking water (*HC + LOS*). Data are expressed as means \pm SE. * $P < 0.05$ vs. control.

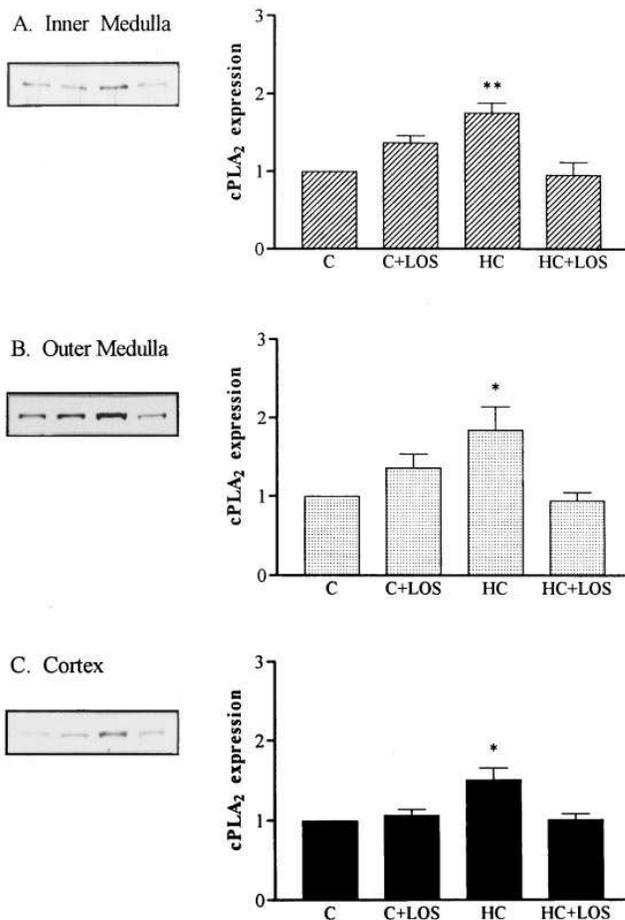


Figure 8. Effect of losartan on cPLA₂ protein expression in hypercalcemia. Representative Western blots (*left*) and densitometric analyses (*right*) demonstrating results obtained in rats fed the control diet alone (*C*), the control diet with losartan in the drinking water (*C + LOS*), the DHT-containing diet for 3 d (*HC*), and the DHT-containing diet and losartan in the drinking water for 3 d (*HC + LOS*). (*A*) Inner medulla (5 μ g protein/well), (*B*) outer medulla (10 μ g protein/well), and (*C*) cortex (20 μ g protein/well). * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control.

report that PGHS-2 protein expression was significantly and maximally increased in hypercalcemic animals after 3 d of ingestion of DHT-containing diet, and displayed a similar cellular pattern of distribution to cPLA₂. Unlike cPLA₂, however, PGHS-2 protein levels in the inner and outer medulla decrease after the initial stimulation on day 3, suggesting that cPLA₂, which is still stimulated at this time, is responsible for increasing PGE₂ production in these regions. In the cortex of hypercalcemic animals, however, levels of PGHS-2 remained significantly elevated until day 5, suggesting that the presence of additional regulatory signals or factors for PGHS-2 expression (in the macula densa and cortical thick ascending limb cells) differ from those influencing interstitial cells of the inner and outer medulla.

PGHS-1 is expressed constitutively in almost all tissues including kidney (15, 16). In this work, we found that PGHS-1 protein levels remained unchanged with hypercalcemia in all kidney regions. Previous studies reveal that PGHS-1 functions

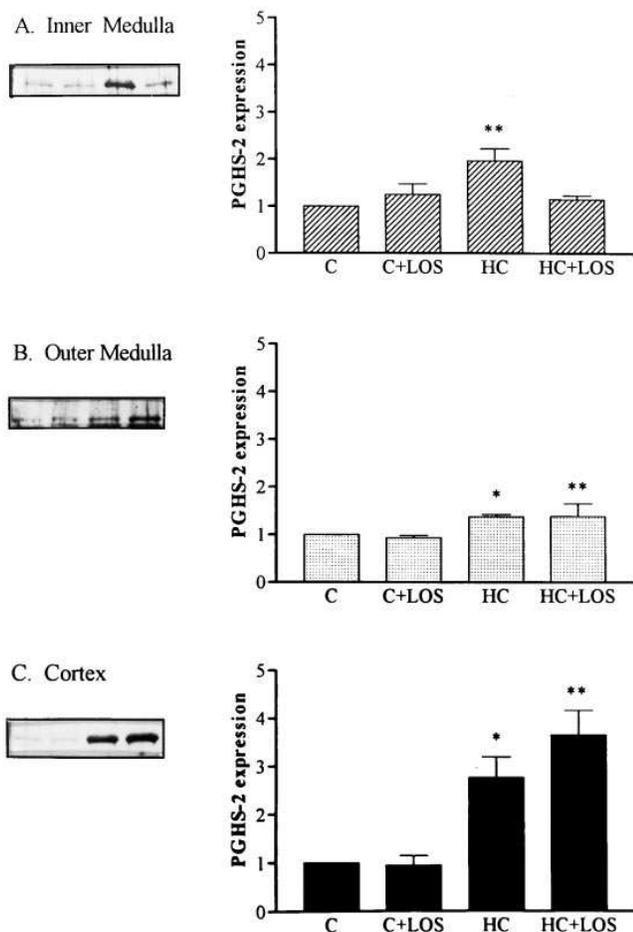


Figure 9. Effect of losartan on PGHS-2 protein expression in hypercalcemia. Representative Western blots (*left*) and densitometric analyses (*right*) of protein expression obtained from rats fed the control diet alone (C), the control diet with losartan in the drinking water (C + LOS), the DHT-containing diet (HC), and the DHT-containing diet and losartan in the drinking water (HC + LOS) for 3 d. (A) Inner medulla (5 μ g protein/well), (B) outer medulla (10 μ g protein/well), and (C) cortex (20 μ g protein/well). * P < 0.05 vs. control; ** P < 0.01 vs. control.

primarily as a housekeeping protein (14). Thus, it appears that in hypercalcemia, PGHS-1 preserves its role as a constitutive protein, responsible for the basal production of prostaglandins, which are most likely involved in the rapid response to stimulation by agonists.

The effects observed in this study are likely due to increased plasma calcium levels, and not due to direct effects of vitamin D in the kidney. Receptors for vitamin D have been localized predominantly to the distal convoluted tubule, connecting segment, initial cortical collecting duct, and proximal tubule (38), regions in which we detected no appreciable immunoreactivity for cPLA₂ or PGHS-2.

Ang II has been reported to stimulate PGE₂ production in kidney tubular epithelial cells, mesangial cells, and interstitial cells (26, 27). Siragy and Carey recently demonstrated that losartan, an AT₁ receptor antagonist, blocked the increase in renal interstitial fluid PGE₂ levels resulting from both sodium

depletion and administration of Ang II (28). In vascular smooth muscle cells, Ang II induces phosphorylation of PLA₂ and increases its activity (39). We investigated the possibility that AT₁ receptor activation occurs in hypercalcemia, and mediates the observed effects. Our data strongly support this hypothesis. First, these studies demonstrate that increased water intake, which is consistently observed in hypercalcemic rats (5, 8, 40), was abolished by administration of losartan, suggesting that polydipsic behavior in hypercalcemia is mediated by binding of Ang II to the AT₁ receptor. Second, losartan prevented the increase in cPLA₂ expression in hypercalcemic animals in all kidney regions, and inhibited increased PGHS-2 expression in the inner medulla without affecting expression in the cortex and outer medulla. Finally, we demonstrate that losartan completely inhibited the increase in urinary PGE₂ excretion observed in rats with hypercalcemia for 5 d. We did not observe any change in urinary PGE₂ levels on day 3, a time when both cPLA₂ and PGHS-2 were stimulated. Thus, there appears to be a lag time between stimulation of expression of these enzymes and the appearance of increased urinary excretion of PGE₂. Previous studies have established that urinary levels of PGE₂ reflect intrarenal synthesis, derived mainly from the inner medulla (41). Taken together, these data suggest that stimulation of cPLA₂ expression, through Ang II activation of the AT₁ receptor in the inner medulla, largely mediates increased PGE₂ excretion in hypercalcemic animals.

Plasma renin activity is not increased significantly in hypercalcemic rats allowed free access to water (5, 6). Furthermore, our data indicate that intrarenal Ang II levels, measured at a time when expression of cPLA₂ and PGHS-2 was increased, are also unchanged with hypercalcemia. This finding suggests that affinity of Ang II for AT₁ receptors or AT₁ receptor number may be stimulated with hypercalcemia. Our data do not, however, permit precise intrarenal localization of sites of action of Ang II in hypercalcemia. Specific binding sites for Ang II (42) and AT₁ receptor mRNA (43) have been detected in all tubular segments, suggesting that Ang II may bind to receptors on cortical and medullary thick ascending limb segments, and

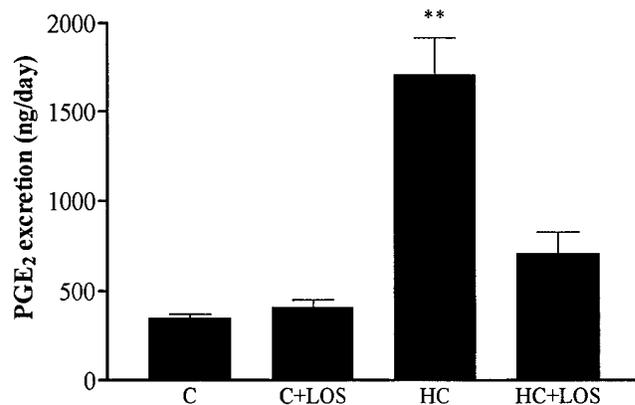


Figure 10. Effect of losartan on PGE₂ excretion. Urine was collected over a 24-h period (day 4 to day 5) from rats fed the control diet (C) alone, the control diet with losartan in the drinking water (C + LOS), the DHT-containing diet (HC), and the DHT-containing diet and losartan in the drinking water (HC + LOS). PGE₂ was measured by radioimmunoassay as described in Methods. Data are expressed as means \pm SE. ** P < 0.001 vs. C, C + LOS, and HC + LOS.

thereby directly stimulate cPLA₂ protein expression. We cannot, however, rule out the possibility that Ang II mediates its effects in an indirect fashion by altering intrarenal hemodynamics.

In this study, cPLA₂ immunoreactivity was detected in interstitial cells of the inner and outer medulla. Zhou et al. have detected Ang II binding sites in type-1 interstitial cells of the inner stripe of the outer medulla, by electron microscopic emulsion autoradiography (44). By autoradiography, this group was unable to detect Ang II binding sites in the inner medulla, although AT₁ Ang II receptor mRNA has been localized to inner medullary collecting duct by reverse transcription PCR (43). Thus, since we also localized cPLA₂ to cells of the inner medullary collecting duct, it is possible that intrarenal expression of this protein with hypercalcemia is partly due to AT₁ receptor stimulation on inner medullary collecting duct cells. Similarly, although PGHS-2 protein expression was stimulated in all regions of the kidney by hypercalcemia in this study, losartan abrogated only the expression in the inner medulla. Since PGHS-2 was immunolocalized only to interstitial cells of the inner medulla, the results suggest the possibility that these cells do indeed contain AT₁ Ang II receptors, or that alteration in renal blood flow mediates the effects of Ang II.

In summary, hypercalcemia upregulates intrarenal cPLA₂ and PGHS-2 protein expression, two proteins critical in the synthesis of prostaglandins. Our data suggest that enhanced expression of cPLA₂ in the inner medulla may account for the elevated urinary PGE₂ levels in hypercalcemia. Furthermore, we have demonstrated an important role for Ang II, acting on AT₁ receptors, in mediating hypercalcemia-induced stimulation of intrarenal cPLA₂ and PGHS-2 expression, and in increasing renal PGE₂ production.

Acknowledgments

We gratefully acknowledge Dr. F.H.H. Leenen and Roselyn White for performing HPLC measurements, and Dr. R. Nair for advice on statistics.

This work was funded by grants from the Medical Research Council of Canada, MT12202 (to L.N. Peterson) and MT1156 (to K.D. Burns). H. Mangat is a recipient of an Ontario Graduate Scholarship. Dr. Burns is a recipient of a Scholarship from the Medical Research Council of Canada.

References

1. Cohen, S.I., M.G. Fitzgerald, P. Fourman, W.J. Griffiths, and H.E. de Wardener. 1957. Polyuria in hyperparathyroidism. *QJM*. 26:423-431.
2. Gill, J.R., and F.C. Bartter. 1961. On the impairment of renal concentrating ability in prolonged hypercalcemia and hypercalcuria in man. *J. Clin. Invest.* 40:716-722.
3. Epstein, F.H., D. Beck, F.A. Carone, H. Levitin, and A. Manitius. 1959. Changes in renal concentrating ability produced by parathyroid extract. *J. Clin. Invest.* 38:1214-1221.
4. Epstein, F.H., M.J. Rivera, and F.A. Carone. 1958. The effect of hypercalcemia induced by calciferol upon renal concentrating ability. *J. Clin. Invest.* 37:1702-1709.
5. Peterson, L.N. 1990. Vitamin D-induced chronic hypercalcemia inhibits thick ascending limb NaCl reabsorption in vivo. *Am. J. Physiol.* 259:F122-F129.
6. Levi, M., L. Peterson, and T. Berl. 1983. Mechanism of concentrating defect in hypercalcemia. Role of polydipsia and prostaglandins. *Kidney Int.* 23:489-497.
7. Serros, E.R., and M.A. Kirschenbaum. 1981. Prostaglandin-dependent polyuria in hypercalcemia. *Am. J. Physiol.* 241:F224-F230.
8. Peterson, L.N., A.J. McKay, and J.S. Borzecki. 1993. Endogenous prostaglandin E₂ mediates inhibition of rat thick ascending limb Cl reabsorption in

chronic hypercalcemia. *J. Clin. Invest.* 91:2399-2407.

9. Farman, N., P. Pradelles, and J.P. Bonvalet. 1986. Determination of prostaglandin E₂ synthesis along rabbit nephron by enzyme immunoassay. *Am. J. Physiol.* 251:F238-F244.

10. Farman, N., P. Pradelles, and J.P. Bonvalet. 1987. PGE₂, PGF₂, 6-keto-PGF₁ and TxB₂ synthesis along the rabbit nephron. *Am. J. Physiol.* 252:F53-F59.

11. Pitcock, J.A. 1988. Morphometric studies of the renomedullary interstitial cells. *Am. J. Med. Sci.* 295:246-251.

12. Gronich, J.H., J.V. Bonventre, and R.A. Nemenoff. 1990. Purification of a high molecular mass form of phospholipase A₂ from rat kidney activated at physiological calcium concentrations. *Biochem. J.* 271:37-43.

13. Smith, W.L., and D.L. DeWitt. 1995. Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. *Semin. Nephrol.* 15:179-194.

14. Langenbach, R., S.G. Morham, H.F. Tiano, C.D. Loftin, B.I. Ghannayem, P.C. Chulada, J.F. Mahler, C.A. Lee, E.H. Goulding, K.D. Kluckman, et al. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell.* 83:483-492.

15. Harris, R.C., J.A. McKanna, Y. Akai, H.R. Jacobson, R.N. Dubois, and M.D. Breyer. 1994. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J. Clin. Invest.* 94:2504-2510.

16. Smith, W.L., and T.G. Bell. 1978. Immunohistochemical localization of the prostaglandin-forming cyclooxygenase in renal cortex. *Am. J. Physiol.* 235:F451-F457.

17. Kujubu, D.A., B.S. Fletcher, B.C. Varnum, R.W. Lim, and H.R. Herschman. 1991. TIS10, a phorbol ester tumor promoter inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* 266:12866-12872.

18. Kujubu, D.A., S.T. Reddy, B.S. Fletcher, and H.R. Herschman. 1993. Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. *J. Biol. Chem.* 268:5425-5430.

19. O'Banion, M.K., V.D. Winn, and D.A. Yung. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA.* 89:4888-4892.

20. Habib, A., C. Creminon, Y. Frobert, J. Grassi, P. Pradelles, and J. Maclouf. 1993. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J. Biol. Chem.* 268:23448-23454.

21. Lee, S.H., E. Soyoola, P. Chanmugam, S. Hart, W. Sun, H. Zhong, S. Liou, D. Simmons, and D. Hwang. 1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.* 267:25934-25938.

22. Kline, R.L., and F. Liu. 1994. Modification of pressure natriuresis by long-term losartan in spontaneously hypertensive rats. *Hypertension (NY)*. 24:467-473.

23. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

24. Bachmann, S., H.M. Bosse, and P. Mundel. 1995. Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. *Am. J. Physiol.* 268:F885-F898.

25. Ruzicka, M., V. Skarda, and F.H.H. Leenen. 1995. Effects of ACE inhibitors on circulating versus cardiac angiotensin II in volume overload-induced cardiac hypertrophy in rats. *Circulation.* 92:3568-3573.

26. Douglas, J.G., and U. Hopfer. 1994. Novel aspect of angiotensin receptors and signal transduction in the kidney. *Annu. Rev. Physiol.* 56:649-669.

27. Zusman, R.M., and H.R. Keiser. 1980. Regulation of prostaglandin E₂ synthesis by angiotensin II, potassium, osmolality, and dexamethasone. *Kidney Int.* 17:277-283.

28. Siragy, H.M., and R.M. Carey. 1996. The subtype-2 (AT₂) angiotensin receptor regulates renal cyclic guanosine 3',5'-monophosphate and AT₁ receptor-mediated prostaglandin E₂ production in conscious rats. *J. Clin. Invest.* 97:1978-1982.

29. Yosipiv, I.V., and S.S. el-Dahr. 1996. Activation of angiotensin-generating systems in the developing rat kidney. *Hypertension (NY)*. 27:281-286.

30. Kaji, D.M., H.S. Chase, Jr., J.P. Eng, and J. Diaz. 1996. Prostaglandin E₂ inhibits Na-K-2Cl cotransport in medullary thick ascending limb cells. *Am. J. Physiol.* 271:C354-C361.

31. Mukherjee, A.B., L. Miele, and N. Pattabiraman. 1994. Phospholipase A₂ enzymes: regulation and physiological role. *Biochem. Pharmacol.* 48:1-10.

32. Schramek, H., Y. Wang, M. Konieczkowski, M.S. Simonson, and M.J. Dunn. 1994. Endothelin-1 stimulates cytosolic phospholipase A₂ activity and gene expression in rat glomerular mesangial cells. *Kidney Int.* 46:1644-1652.

33. Brown, C.A., R.M. Zusman, and E. Haber. 1980. Identification of an angiotensin receptor in rabbit renomedullary interstitial cells in tissue culture. *Circ. Res.* 46:802-807.

34. Kester, M., E. Coroneos, P.J. Thomas, and M.J. Dunn. 1994. Endothelin stimulates prostaglandin endoperoxide synthase-2 mRNA expression and protein synthesis through a tyrosine kinase-signaling pathway in rat mesangial cells. *J. Biol. Chem.* 269:22574-22580.

35. Mitchell, J.A., S. Larkin, and T.J. Williams. 1995. Cyclooxygenase-2: regulation and relevance in inflammation. *Biochem. Pharmacol.* 50:1535–1542.
36. Wu, K.K. 1996. Cyclooxygenase 2 induction: molecular mechanism and pathophysiologic roles. *J. Lab. Clin. Med.* 128:242–245.
37. DeWitt, D.L., and E.A. Meade. 1993. Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. *Arch. Biochem. Biophys.* 306:94–102.
38. Liu, L., M. Ng, A.M. Iacopino, S.T. Dunn, M.R. Hughes, and J.E. Bourdeau. 1994. Vitamin D receptor gene expression in mammalian kidney. *J. Am. Soc. Nephrol.* 5:1251–1258.
39. Rao, G.N., B. Lassegue, R.W. Alexander, and K.K. Griendling. 1994. Angiotensin II stimulates phosphorylation of high-molecular-mass cytosolic phospholipase A2 in vascular smooth-muscle cells. *Biochem. J.* 299:197–201.
40. Kraly, S.F., and R. Corneilson. 1990. Angiotensin II mediates drinking elicited by eating in the rat. *Am. J. Physiol.* 258:R436–R442.
41. Taverner, D., R.F. Bing, A. Fletcher, G. Russell, J.D. Swales, and H. Thurston. 1984. Hypertension produced by chemical renal medullectomy: evidence for a renomedullary vasodepressor function in the rat. *Clin. Sci. (Lond.)* 67:521–528.
42. Mujais, S.K., S. Kauffman, and A.I. Katz. 1986. Angiotensin II binding sites in individual segments of the rat nephron. *J. Clin. Invest.* 77:315–318.
43. Terada, Y., K. Tomita, H. Nonoguchi, and F. Marumo. 1993. PCR localization of angiotensin II receptor and angiotensinogen mRNAs in rat kidney. *Kidney Int.* 43:1251–1259.
44. Zhuo, J., D. Alcorn, J. McCausland, and F.A. Mendelsohn. 1994. Localization and regulation of angiotensin II receptors in renomedullary interstitial cells. *Kidney Int.* 46:1483–1485.