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Inhibitory Effect of Potassium Citrate on Rat Renal Tumors Induced by N-Ethyl-N-hydroxyethylnitrosamine Followed by Potassium Dibasic Phosphate

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Potassium dibasic phosphate (PDP) was administered at a concentration of 10% by weight in basal diet to unilaterally nephrectomized Wistar rats previously given 1000 ppm N-ethyl-N-hydroxyethylnitrosamine (EHEN) in the diet for 2 weeks. To study the effect of alkalinization on renal mineralization, some animals concomitantly received 5% potassium citrate (PC). Feeding PDP alone promoted adenomatous hyperplasias, which were regarded as preneoplastic lesions, as well as renal cell tumors in EHEN-initiated rats, whereas the addition of PC to PDP diets reduced the promoting effect. Histopathology, serum biochemistry and urinalysis indicated retardation of renal calcium crystallization by PC. Two other phosphate salts, sodium phosphate (SP) and calcium phosphate (CP), were also administered. SP showed a slight promoting effect on adenomatous hyperplasias and a 2-fold increase in the yield of renal cell tumors, while CP induced a clear reduction of both lesions, over EHEN alone. The promoting effects of both PDP and SP and the inhibitory effect of PC were somewhat correlated to 5-bromo-2′-deoxyuridine labeling indices, the degree of nephropathy, and mineralization in the kidney. Immunohistochemically, the nephropathy induced by phosphate salts was not linked to α_{2u}-globulin. A pathogenesis for renal carcinogenesis is suggested in which nephropathy associated with mineralization enhances the development of renal cell tumors.

Key words: Rat renal tumor — Potassium citrate — Inhibition — Nephropathy

Among environmental exposures associated with cancer, nephrotoxic agents are one of the most important factors in renal carcinogenesis.[1–5] Although many mechanisms for carcinogenesis exist, sustained increase in cell proliferation of renal tubular epithelium may prove to be an essential component of the carcinogenic response to nongenotoxic nephrotoxins. Induction of renal tumors in animals provides models for investigating the pathogenesis and mechanisms of renal carcinogenesis. For example, a male rat-specific renal disease, known as α_{2u}-globulin nephropathy, leads to degeneration, necrosis of the proximal tubular epithelium and subsequent increased rates of cell proliferation.[6–8] Chronic exposure to α_{2u}-globulin results low but significant incidence of renal tumors in male rats.[9, 10] A similar pathogenesis may be hypothesized for tumor promotion by some other nongenotoxic nephrotoxins. EHEN[2] is a carcinogen which selectively causes renal epithelial tumors. We have previously reported the possible application of a medium-term bioassay for detection of renal carcinogenesis in unilaterally nephrectomized rats initiated with EHEN.[11] We have also recently demonstrated the promoting effect of PDP on early-stage renal carcinogenesis in the same bioassay system.[12] The treatment with PDP induced renal mineralization and promoted the development of preneoplastic and neoplastic lesions in rat kidneys. Although some salt mixtures, including PDP, are known to induce nephrotoxic effects in experimental animals, the mechanism of promotion by PDP is still not clear. The purpose of the present study was to investigate the nephrocarcinogenicity and nephrotoxicity through the mediation of renal mineralization induced by various salts, using our medium-term bioassay. Moreover, we examined whether or not the prevention of PDP-induced nephropathy by calcium reduction prohibits the promotion of renal tumorigenesis initiated by EHEN.

MATERIALS AND METHODS

Chemicals and diet EHEN [CAS: [3]47-25-6, 2-(ethylnitrosamine)ethanol, purity 99.8%, liquid at room temperature], PDP, PC, SP and CP (purity >99+%) were purchased from Nakarai Chemical Ltd., Kyoto. The latter four compounds were mixed in basal diet (Oriental M powder, Oriental Yeast Co., Osaka) at concentrations of 10%, 5%, 10% and 10%, respectively.

Animals and experimental design A total of 200 male Wistar rats (Std:Wistar) were purchased at 6 weeks of age from Nihon SLC (Shizuoka) and acclimated on basal diet and tap water *ad libitum* for 1 week. All rats were housed in wire-bottomed cages at a room temperature of

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[2] Abbreviations: EHEN, N-ethyl-N-hydroxyethylnitrosamine; PC, potassium citrate; PDP, potassium dibasic phosphate; SP, sodium phosphate; CP, calcium phosphate; BrdU, 5-bromo-2′-deoxyuridine.

24±1°C and with 55±5% relative humidity. At 7 weeks of age, they were divided into 10 groups; animals in groups 1, 3, 5, 7 and 9 received 1000 ppm EHEN in the diet for 2 weeks while groups 2, 4, 6, 8 and 10 received the basal diet only. At week 3, all animals underwent a unilateral nephrectomy of the left kidney and were subsequently placed on diets containing the salts as follows: groups 1 and 2, 10% PDP; groups 3 and 4, 10% PDP + 5% PC; groups 5 and 6, 10% SP; groups 7 and 8, 10% CP. Groups 9 and 10 remained on basal diet without additives. The duration of salt feeding was 18 weeks.

Clinical parameters Whole body, liver and kidney weights were recorded for all animals at termination of the experiment at 20 weeks. Urine samples were collected in tubes containing a small amount of sodium azide over an 8 h period from 5 randomly selected rats in each experimental group. Blood for serum biochemistry was taken from the same representative animals from each group under ether anesthesia immediately prior to death.

Monoclonal antibody to α_{2u}-globulin α_{2u}-Globulin was isolated and purified from male Wistar rat urine by a modification of the method of Kurtz *et al.*[13] A mouse monoclonal IgG antibody to α_{2u}-globulin was produced by the method previously reported.[14] Specificity for rat α_{2u}-globulin was demonstrated by Western blot analysis, which confirmed the presence of a single band of immunoreactive product of 18,700 daltons.

Pathology and immunohistochemistry Five animals from each experimental group were given a single i.p. injection of 100 mg/kg body weight of BrdU (Sigma Chemical Co., St. Louis, MO) 1 h prior to death. All animals were killed under ether anesthesia, and body, liver and kidney weights were recorded as mentioned previously. The kidneys and samples of small intestine from BrdU-treated rats were fixed for 24 h in 70% ethanol and processed using the avidin-biotin-peroxidase complex (ABC) technique for immunohistochemical studies of cell proliferation and DNA synthesis.[15] BrdU-labeled cells in renal cortical tubular epithelia were counted in each kidney in areas of 1 mm^2 free of preneoplastic lesions. Organs from remaining animals were preserved in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for histologic evaluation.

The terms employed in the histological classification of lesions merit explanation. In this and in our earlier studies, we have used the terms simple hyperplasia, adenomatous hyperplasia and renal cell tumor; this latter term includes both adenomas and carcinomas. There are many synonymous terms for the same morphologic entities.[16] We use simple hyperplasia to reflect the often reversible, non-neoplastic cell proliferation induced by nephrotoxins, while adenomatous hyperplasia is considered to be a possible preneoplastic lesion. Thus, the numbers of simple hyperplasia, adenomatous hyperplasia, and renal cell tumors (which includes both adenomas and carcinomas) per rat were counted in 4 sagittal sections of each kidney. Additional kidney sections from each group were immunohistochemically stained with monoclonal antibody against α_{2u}-globulin. Quantitative data were analyzed for statistical significance by using Student's t test for multiplicity and the chi-square test for incidence.

RESULTS

Body and kidney weights The mean body weights at 20 experimental weeks in each of the treated groups were significantly lower than in the untreated control, group 10 (Table I). Groups 1–4 also showed significantly lower weights than those of group 9 (EHEN alone). However, the relative kidney-to-body weight ratios, expressed as %, for groups 1–5 showed significant increases over those of groups 9 and 10.

Serum biochemistry and analysis The results of serum biochemistry are shown in Table II. Statistically significant increases ($P<0.05$) were observed in the blood urea nitrogen (BUN), creatinine (Cr), sodium (Na), potassium (K) and phosphate (P) values of PDP-treated rats relative to controls, with the exception of BUN in group 4. Significant decreases were noted in calcium (Ca) and chloride (Cl) in the same groups, again except for Ca levels in group 4. It is interesting to note that the addition of PC seems to allow some recovery of the Ca values reduced by PDP feeding.

There was a general decrease in all urine parameters measured in PDP-treated rats as shown in Table III, while addition of PC to the diet had no discernible effect. Urinary pH was elevated to between 8.0 and 8.4 in both PDP- and CP-treated animals.

BrdU labeling indices The numbers of BrdU-labeled cells counted in 1 mm^2 fields of normal renal cortex per treatment group are recorded in Table IV. Exposure to either PDP or SP significantly elevated the cortical labeling indices over controls; concomitant PDP and PC feeding reduced the elevation in numbers of BrdU-positive cells over control values, compared to that observed in groups receiving PDP alone.

Histopathology and α_{2u}-globulin immunohistochemistry The degree of nephropathy, based on previously published criteria,[17] and the relative degree of renal mineralization are also summarized in Table IV. Nephropathy and mineralization were observed in PDP- and SP-treated groups and the areas of nephropathy were closely associated with areas of mineralization (Fig. 1). Rats fed CP showed renal hyperplasia without degenerative nephropathy or mineralization. The number of rats demonstrating renal lesions (incidence) and the numbers of lesions per affected rat (multiplicity) are shown in

Table I. Body and Kidney Weights of Nephrectomized Rats Treated with EHEN and Test Chemicals (20 Weeks)

Group	Treatment	Weight (g)		KW/BW (%)
		Body (BW)	kidney (KW)	
1	EHEN→10%PDP	235.5±41.5[a, b]	1.56±0.15	0.67±0.09[a, b]
2	BD →10%PDP	217.8±29.2[a, b]	1.50±0.21	0.70±0.12[a, b]
3	EHEN→10%PDP+5%PC	186.7±31.3[a, b, c]	1.39±0.17	0.76±0.11[a, b]
4	BD →10%PDP+5%PC	232.9±25.6[a, b]	1.43±0.13	0.62±0.07[a, b]
5	EHEN→10%SP	303.2±24.5[a]	1.60±0.25	0.53±0.07[a, b]
6	BD →10%SP	330.6±24.2[a]	1.63±0.16[b]	0.49±0.04[b]
7	EHEN→10%CP	309.9±30.5[a]	1.42±0.15	0.46±0.04
8	BD →10%CP	287.1±20.5[a, b]	1.22±0.10[a, b]	0.43±0.03
9	EHEN→BD	329.0±29.2[a]	1.46±0.13	0.45±0.03
10	BD (control)	363.6±9.1	1.58±0.24	0.44±0.07

BD: basal diet.
a) Significantly different from group 10 ($P<0.05$).
b) Significantly different from group 9 ($P<0.05$).
c) Significantly different from group 1 ($P<0.05$).

Table II. Serum Biochemistry Values of Nephrectomized Rats Treated with EHEN and Test Chemicals (20 Weeks)

Group	Treatment	BUN	Cr	Na	K	Ca	Cl	P
1	EHEN→10%PDP	44.1[a]	1.56[a]	147.0[a]	5.86[a]	3.26[a]	94.9[a]	13.5[a]
2	BD →10%PDP	60.6[a]	1.98[a]	149.8[a]	5.81[a]	3.03[a]	93.6[a]	17.1[a]
3	EHEN→10%PDP+5%PC	62.6[a]	1.57[a]	146.4[a]	5.77	3.73[a]	97.1[a]	10.5[a]
4	BD →10%PDP+5%PC	27.5[b]	1.11[a, b]	148.0[a]	6.16[a]	4.81[b]	97.4[a]	10.0[a]
5	EHEN→10%SP	44.9	1.21	144.8	4.42[b]	4.79[b]	102.8[b]	6.6[b]
6	BD →10%SP	24.8[b]	0.82[b]	144.9	4.62[b]	4.75[b]	100.6[a, b]	7.5[b]
7	EHEN→10%CP	30.0[b]	0.83[b]	144.1[b]	4.60[b]	5.11[b]	101.2[b]	6.5[b]
8	BD →10%CP	26.6[b]	0.76[b]	144.3	4.69[b]	4.87[b]	102.6[b]	5.3[b]
9	EHEN→BD	26.0[b]	0.68[b]	142.9[b]	4.96[b]	5.04[b]	103.0[b]	6.2[b]
10	BD (control)	27.8[b]	0.70[b]	142.5[b]	4.79[b]	5.00[b]	102.9[b]	5.8[b]

BD: basal diet.
a) Significantly different from group 10 ($P<0.05$).
b) Significantly different from group 1 ($P<0.05$).

Table III. Urine Analysis of Nephrectomized Rats Treated with EHEN and Test Chemicals (20 Weeks)

Group	Treatment	BUN	Cr	Na	K	Ca	Cl	P	pH
1	EHEN→10%PDP	300[a]	13.6[a]	54.8[a]	38.6[a]	1.04[a]	37.8[a]	87.9[a]	8.0[a]
2	BD →10%PDP	288[a]	12.8[a]	46.0[a]	33.4[a]	1.04[a]	32.4[a]	102.8[a]	8.0[a]
3	EHEN→10%PDP+5%PC	200[a]	36.4[a]	46.8[a]	13.6[a, b]	1.26[a]	34.0[a]	87.6[a]	8.1[a]
4	BD →10%PDP+5%PC	168[a]	14.8[a]	47.8[a]	33.0[a]	0.82[a]	28.2[a]	85.4[a]	8.0[a]
5	EHEN→10%SP	1316[a]	109.2	178.0[a]	191.6	3.44[a]	88.6[a]	394.6[a]	6.4
6	BD →10%SP	3388	260.0[a]	450.0[a]	455.2	8.18	207.2	370.0[a]	6.1
7	EHEN→10%CP	1552[a]	101.2	86.0[a]	584.2[a]	7.92	85.2[a]	131.2	8.4[a]
8	BD →10%CP	2112	123.6	126.0	679.0[a]	6.62[a]	112.4	227.4	8.4[a]
9	EHEN→BD	2577	107.8	114.0	294.6	13.32	185.5	144.7	6.7
10	BD (control)	2456	128.2	103.0	284.8	10.86	158.4	193.8	6.2

BD: basal diet.
a) Significantly different from group 10 ($P<0.05$).
b) Significantly different from group 1 ($P<0.05$).

Table IV. BrdU-labeled Cells, Nephropathy and Mineralization in Nephrectomized Rats Treated with EHEN and Test Chemicals

Group	Treatment	BrdU LI (cells/mm^2)	Nephropathy grade	Mineralization[d]
1	EHEN→10%PDP	28.60±3.01[a, b]	3	+++
2	BD →10%PDP	25.20±1.75[a, b]	3	+++
3	EHEN→10%PDP+5%PC	14.50±2.54[a, b, c]	3	++
4	BD →10%PDP+5%PC	16.10±1.94[a, b]	2	++
5	EHEN→10%SP	15.40±4.46[a, b]	3	++
6	BD →10%SP	9.20±2.35[a, b]	2	+
7	EHEN→10%CP	2.92±1.56	0	−
8	BD →10%CP	0.88±0.27[a, b]	0	−
9	EHEN→BD	3.80±0.95	0	−
10	BD (control)	3.15±0.68	0	−

a) Significantly different from group 10 ($P<0.05$).
b) Significantly different from group 9 ($P<0.05$).
c) Significantly different from group 1 ($P<0.05$).
d) +++: severe, ++: moderate, +: mild.
BrdU-labeled cells were counted in renal cortical tubular epithelia without preneoplastic lesions.

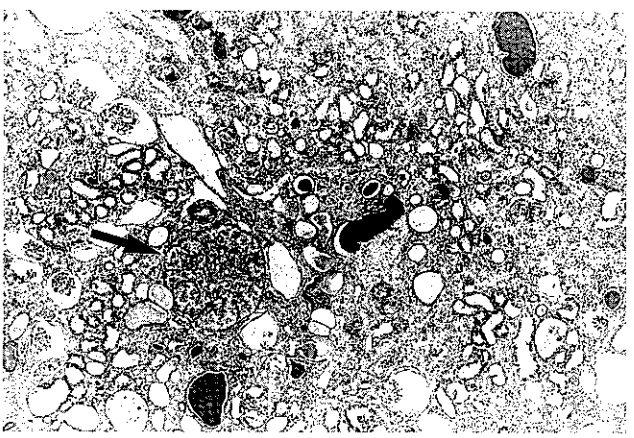

Fig. 1. Severe nephropathy showing scattered dilated tubules with hyaline casts, glomerular sclerosis, lymphocyte infiltration and interstitial fibrosis induced by EHEN and 10% PDP. A renal cell tumor (adenoma) is also seen (arrow). (HE stain, ×40).

Table V. The histological classifications for proliferative renal lesions have been described in previous reports.[11, 12] Simple hyperplasias could not be accurately detected in PDP-treated groups (groups 1–4) because of the extent of severe masking nephropathy. Simple hyperplasia exhibited by chemically untreated controls (group 10) was probably compensatory as a result of the contralateral nephrectomy. All animals in groups 1–5 developed adenomatous hyperplasias, and EHEN exposure increased the multiplicity in groups 1–9 as compared to non-initiated dietary controls. Rats receiving EHEN followed by PDP or SP gave the highest renal cell tumor incidences, i.e., 100% and 53.8%, respectively, as well as the greatest multiplicity of 2.0 and 1.08 tumors per kidney, respectively. Co-administration of PC and PDP to EHEN-initiated animals resulted in a significant decrease of both the incidence and multiplicity of renal tumors compared to the group treated with EHEN followed by PDP only. Magnitude of mineralization was slightly decreased in PDP and PC-treated groups (groups 3 and 4) as compared to PDP-treated groups. Although BrdU labeling index (LI) in group 3 was significantly decreased among these groups, the grades of nephropathy were almost the same. CP appeared to have little impact on the incidence of renal lesions compared to appropriate controls.

No positive reaction to anti-α_{2u}-globulin was observed in any abnormal kidney tissue from any treatment group. However, normal tubular epithelium was occasionally positive (Fig. 2).

DISCUSSION

A number of chemical agents are known to induce renal tumors in rodents,[18] and nongenotoxic nephrotoxins play an important role in renal tumorigenesis. Several nephrotoxic compounds, such as unleaded gasoline, have the ability to induce degenerative nephropathies, which have been linked to an increased incidence of renal adenomas and carcinomas in the rat.[7] A considerable amount of data has accumulated on the relationship between nephrotoxicity and nephrocarcinogenicity. It has become evident that toxic lesions precede and develop concurrently with tumorigenesis.[19] One mecha-

Table V. Incidences of Renal Lesions in Nephrectomized Rats Treated with EHEN and Test Chemicals (20 Weeks)

Group	Treatment	No. of rats with (%)			Multiplicity		
		Simple hyperplasia	Adenomatous hyperplasia	Renal cell tumor	Simple hyperplasia	Adenomatous hyperplasia	Renal cell tumor
1	EHEN→10%PDP	ND	15 (100)[a]	15 (100)[a, b]	ND	4.13±1.27[a, b]	2.00±1.00[a, b]
2	BD →10%PDP	ND	15 (100)[a]	0 (0)	ND	0.92±0.86[a]	0
3	EHEN→10%PDP+5%PC	ND	14 (100)[a]	4 (28.6)[a, c]	ND	2.43±1.50[a]	0.43±0.73[c]
4	BD →10%PDP+5%PC	ND	15 (100)[a]	0 (0)	ND	0.33±0.47[b]	0
5	EHEN→10%SP	13 (100)	13 (100)[a]	7 (53.8)[a]	8.23±1.93[a, b]	4.46±2.41[a, b]	1.08±1.21[a]
6	BD →10%SP	17 (100)	10 (58.8)[a, b]	0 (0)	4.25±1.42[a, b]	0.83±1.07[a]	0
7	EHEN→10%CP	18 (100)	10 (55.6)[a, b]	3 (16.7)	3.56±1.54[a, b]	0.83±0.83[a]	0.17±0.37
8	BD →10%CP	18 (90.0)	4 (20.0)[b]	0 (0)	0.79±0.77[a, b]	0.21±0.41[b]	0
9	EHEN→BD	20 (100)	18 (90.0)[a]	5 (25.0)[a]	3.50±1.41[a]	1.75±1.20[a]	0.25±0.43
10	BD (control)	20 (100)	0 (0)	0 (0)	0.88±0.78[b]	0	0

BD: basal diet, ND: not detectable.
a) Significantly different from group 10 ($P<0.05$).
b) Significantly different from group 9 ($P<0.05$).
c) Significantly different from group 1 ($P<0.05$).

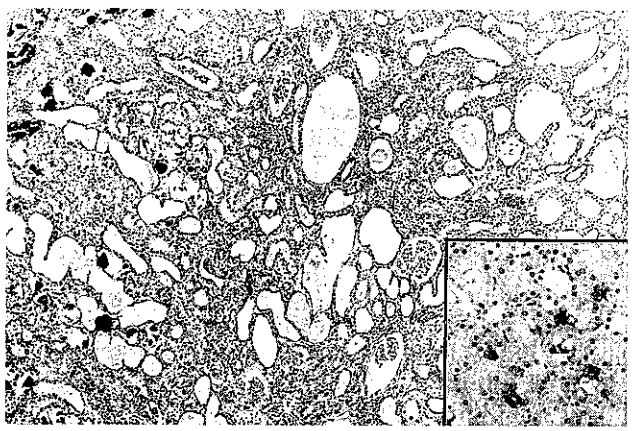

Fig. 2. Negative immunohistochemical staining of α_{2u}-globulin in the kidney induced by EHEN and 10% PDP. Scattered positive tubular cells in the control kidney (insert). (ABC method, counterstained with hematoxylin, ×40).

nism proposed is that of cellular degeneration and death, caused by toxic insult, resulting in an increased cellular proliferation, or hyperplasia, in regenerating tissues. This hyperplastic response may increase the chance of expression of endogenously or exogenously induced mutations in the proliferating cell population, leading to tumor formation.

In the present study, PDP and SP appear to be good promoters of EHEN-initiated kidney neoplasias in the rat. The mechanism of tumor development enhanced by these phosphate salts seems to follow this degeneration/regeneration model as indicated by the histopathological findings, the serum biochemistry and urinalysis. Moreover, a dose-dependent increase in the promotional effects of PDP is evident. Using 10% PDP in this study, the incidence of adenomatous hyperplasia was 6 times higher and the renal cell tumor incidence twice that obtained in an earlier study feeding 5% PDP.[12] Ten per cent PDP also induced BrdU labeling indices 7 times greater than those reported in the aforementioned study, in which animals were given 5% PDP for the same length of time. CP, on the other hand, showed no promotion of renal lesions over controls, and it had little effect on serum or urinary electrolyte values. This may be explained by the fact that CP dissociates less readily than either PDP or SP,[20] and is probably not absorbed to any great extent from the gastrointestinal tract, or, alternatively, it may not be ionizable in the blood once absorbed.

Acute and chronic renal toxicity produced by unleaded gasoline is believed to result from the accumulation of intracellular α_{2u}-globulin droplets in the proximal tubules. This accumulation of protein is associated with increased epithelial cell proliferation.[7, 8, 21] Although PDP- and SP-induced nephropathy does not appear to be related to α_{2u}-globulin accretion, as evidenced by negative immunohistochemistry, a pathogenesis may be hypothesized for tumor promotion, substituting calcium for protein deposition. Intracellular calcium homeostasis is closely related to renal acidosis and cell injury, in that increased calcium accumulation, common in chemically induced toxicity, can cause coagulative necrosis.[22] Human renal cell tumors have been reported in association with renal calcification.[23]

Clinically, patients suffering from distal renal tubular acidosis usually receive alkali therapy using Stohl's solution, which consists of sodium citrate and citric acid. Alkaline solutions made from the sodium salts have,

however, been suggested to increase the risk of calcium stone formation[24, 25]; on the other hand, use of potassium salts has been shown to give more favorable results in tubular acidosis patients by reducing calcium nephrolithiasis.[26, 27] Renal calcification, as well as promotion, by 5% PDP in EHEN-induced renal tumorigenesis studies has been noted previously,[12] and the higher concentration used in the present experiment yielded more severe mineralization. Urine calcium concentrations in groups treated with PDP and SP (groups 1–5) were significantly lower than that of untreated controls. Serum calcium levels in groups 1–3 were also lowered significantly from that of untreated controls; this might be due, at least in part, to calcium phosphate deposition in the kidney. Histologically, addition of PC clearly reduced calcium deposition in the kidney and serum analysis showed slightly, though not significantly, increased calcium levels after PC administration. Citrate also acts to reduce the urinary saturation of calcium phosphate by direct complex formation with the calcium ion.[28] The reduction in adenomatous hyperplasia multiplicity and in the incidence and multiplicity of renal tumors seen in PC-treated groups may thus be a result of reduced BrdU LI, cell proliferation and some reduction in calcification-induced nephropathy. Nutritional influences might produce reduced tumor incidences, though the same inhibitory effect of PC was observed in the 5% PDP group (data not shown). Moreover, the incidences and multiplicities of renal lesions were increased in 10% PDP groups, even if the suppression of body weight gain occurred.

In conclusion, the ability of PDP and SP to promote EHEN-initiated renal tumorigenesis in unilaterally nephrectomized rats appears to be associated with nephropathy and subsequent cell proliferation, which occurs secondary to calcification. α_{2u}-Globulin nephropathy does not play a role in the promotional activity of these phosphate salts. Administration of PC concomitantly with PDP significantly reduced the degree of mineralization, nephropathy, cell proliferation and, ultimately, the promotion of renal preneoplastic and neoplastic lesions induced by PDP. These findings also indicate that, as in humans, mineralization itself may play a significant part in the sequence of events leading to tumor formation in the kidney.

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