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Promoting Effects of Potassium Dibasic Phosphate on Early-stage Renal Carcinogenesis in Unilaterally Nephrectomized Rats Treated with N-Ethyl-N-hydroxyethylnitrosamine

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The effects of potassium dibasic phosphate (PDP), potassium aluminum sulfate (PAS) and copper sulfate (CS) on early-stage renal carcinogenesis were investigated in unilaterally nephrectomized male Wistar rats after N-ethyl-N-hydroxyethylnitrosamine (EHEN) administration. After feeding 1,000 ppm EHEN, or basal diet for 2 weeks and removal of the left kidney at week 3, male Wistar rats were divided into 8 groups of 20 rats each. These groups received the following dietary treatments: 50,000 ppm PDP, 50,000 ppm PAS, 5,000 ppm CS or basal diet, respectively, for 18 weeks from weeks 3 to 20. The average numbers of adenomatous hyperplasias counted as preneoplastic lesions in the EHEN with 50,000 ppm PDP group were significantly higher than in the EHEN alone group or the EHEN followed by 50,000 ppm PAS or 5,000 ppm CS group. The treatment with 50,000 ppm PDP induced renal calcification and promoted the development of preneoplastic lesions in unilaterally nephrectomized rats treated with EHEN, but that with 50,000 ppm PAS or 5,000 ppm CS did not.

Key words: N-Ethyl-N-hydroxyethylnitrosamine — Renal tumor — Promoter — Nephrectomy — Potassium dibasic phosphate

Nephrotoxic chemicals have been reported to promote the development of renal cell tumors in rats treated with chemical carcinogens.[1-10] In the majority of cases, an experimental period of more than 30 weeks proved necessary for detection of promoting effects in terms of renal cell tumor development.

Recently, the possible application of medium-term organ bioassays to detection of renal carcinogenesis modifiers has been reported.[11, 12] It could be confirmed that nephropathy induced by various factors[1-13] is associated with promoting potential, but it was also found that diethylene glycol, which is a nephrotoxic chemical, did not enhance the development of renal cell tumors in rats treated with EHEN[2] and nephrectomy.[11] Thus the correlation between toxicity and modulation is not perfect.

PDP, PAS and CS are needed as salts in the diet. Deficiency of PDP, PAS and CS is known to induce disease but no toxicity of PDP, PAS and CS has so far been reported. In a preliminary experiment more than 20 times the normal doses of various salts including PDP, PAS and CS were tested for toxicity over 8 weeks. PDP but not PAS and CS caused increase in kidney weight and induced calcification. Slight deposits of material were found in the proximal convoluted tubular cells and lumen after PAS and CS administration. No other salts caused any alterations and therefore PDP, PAS and CS were chosen for investigation at high doses (maximum 50,000 ppm) in the present study on the basis of the preliminary experiment.

The purpose of the present studies was to apply our medium-term organ bioassay for renal carcinogenesis modifiers to PDP, PAS and CS, which all induce renal injury.

MATERIALS AND METHODS

Chemicals and diet EHEN [CAS: 13147-25-6, 2-ethylnitrosamine (ethanol), purity, 99.8, liquid at room temperature] was purchased from Nakarai Chemical, Ltd., Kyoto. PDP, PAS and CS were purchased from Nakarai Chemical Ltd. and incorporated into basal diet (Oriental M; powder, Oriental Yeast Co., Osaka) by admixture with a blender.

Animals and experimental design A total of 165 male inbred 6-week-old Wistar rats were purchased from Nihon SLC (Shizuoka) and maintained under constant conditions on basal diet and tap water *ad libitum*. After 1 week, 160 healthy rats, weighing 140–160 g, were divided into 8 groups and given the following diets; group I (20 rats) was given 1,000 ppm EHEN diet for 2 weeks and then 50,000 ppm PDP diet for 18 weeks; group II (20 rats) received the basal diet for 2 weeks and then 50,000 ppm PDP diet for 18 weeks; group III received 1,000 ppm EHEN diet for 2 weeks and then 50,000 ppm PAS diet for 18 weeks; group IV received the

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[2] Abbreviations: EHEN, N-ethyl-N-hydroxyethylnitroamine; PDP, potassium dibasic phosphate; PAS, potassium aluminum sulfate; CS, copper sulfate; BrdU, bromodeoxyuridine.

basal diet for 2 weeks and then 50,000 ppm PAS diet for 18 weeks; group V received 1,000 ppm EHEN diet for 2 weeks and then 5,000 ppm CS diet; group VI received the basal diet for 2 weeks and then 5,000 ppm CS diet; group VII received 1,000 ppm EHEN diet for 2 weeks and then basal diet for 18 weeks; and group VIII received the basal diet for 20 weeks. All animals were unilaterally nephrectomized at the biginning of week 3 and killed at the end of the experiment at week 20. BrdU was injected ip at 2 mg per 100 g body weight into all rats one hour before death. Blood was collected from all rats under ether anesthesia immediately prior to death and used for chemical assays of electrolytes. All livers and kidneys were immediately excised, weighed, fixed with 70% ethanol and processed routinely for histological and immunohistochemical studies. The numbers of simple hyperplastic foci,[11] adenomatous hyperplastic foci[11] and renal cell tumors[11] per rat were counted in four sagittal sections of each kidney. BrdU-labeled tubular cells per mm^2 were counted histochemically in randomly selected areas with and without preneoplastic and neoplastic lesions in the renal cortex ten times in each kidney. Quantitative data were analyzed for statistical significance by student's t test for frequency and the chi-square test for incidence.

RESULTS

Average body and kidney weights Data for body and kidney weights in each group are shown in Table I. The mean final body weights in groups I, II, III, V and VI were significantly lower than that in group VIII. The mean relative kidney weight per body weight in group I was significantly higher than those in groups VII and VIII. The mean kidney weight in group II was significantly higher than that in group VIII.

Serum electrolytes The mean values for serum electrolytes in each group are shown in Table II, no significant intergroup differences being apparent.

Incidences of simple hyperplastic foci, adenomatous hyperplastic foci and renal cell tumors Cells of simple hyperplastic foci can be divided into two types, i.e., apparently neoplastic cells and regenerating cells which are in a reversible state. Simple hyperplastic foci were diagnosed for lesions maintaining a tubular pattern with lumen and adenomatous hyperplastic foci for lesions without tubular patterns.

Incidences of rats with simple hyperplastic foci (Fig. 1), adenomatous hyperplastic foci (Fig. 2) and renal cell tumors (Figs. 2 and 3) are summarized in Table III. Incidences of rats with simple hyperplastic foci were 100% (20/20) in group I, 45% (9/20) in group II, 70%

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

Group	Treatment	Body weight (g)	Kidney/body weight (%)
I	EHEN+5%PDP	296±20[b]	0.55±0.05[a, b]
II	5%PDP	309±17[b]	0.54±0.06[b]
III	EHEN+5%PAS	307±13[b]	0.42±0.04
IV	5%PAS	320±20	0.41±0.02
V	EHEN+0.5%CS	290±24[a, b]	0.42±0.02
VI	0.5%CS	302±23[b]	0.41±0.02
VII	EHEN	322±30	0.44±0.01
VIII	Control	339±32	0.45±0.06

EHEN, N-ethyl-N-hydroxyethylnitrosamine; PDP, potassium dibasic phosphate; PAS, potassium aluminum sulfate; CS, copper sulfate.
a) Significantly different ($P<0.05$) from group VII.
b) Significantly different ($P<0.05$) from group VIII.

Table II. Serum Electrolytes in Nephrectomized Rats Treated with EHEN and Test Chemicals (20 Weeks)

Group	Treatment	Electrolytes (mEq/liter)					
		Na	K	Cl	Ca	P	Mg
I	EHEN+5%PDP	142.2±1.6	4.7±0.3	97.5±1.1	5.0±0.2	5.7±1.1	1.9±0.4
II	5%PDP	141.9±1.6	4.7±0.4	99.4±1.6	4.9±0.2	5.5±0.6	1.9±0.2
III	EHEN+5%PAS	142.5±0.5	4.8±0.3	101.3±0.8	5.1±0.1	6.6±0.4	2.2±0.1
IV	5%PAS	141.5±1.1	4.7±0.3	100.8±0.4	5.0±0.1	5.9±0.4	1.9±0.2
V	EHEN+0.5%CS	143.0±2.5	4.9±0.4	99.0±1.6	5.2±0.6	5.9±1.1	2.0±1.0
VI	0.5%CS	142.0±1.0	4.9±0.0	96.0±1.0	5.2±0.2	5.5±0.3	2.0±0.1
VII	EHEN	141.5±1.1	4.5±0.2	98.3±1.5	5.2±0.1	6.2±0.4	2.2±0.3
VIII	Control	142.3±3.6	5.4±1.0	102.0±2.2	5.7±0.6	7.3±2.0	2.4±1.0

EHEN, N-ethyl-N-hydroxyethylnitrosamine; PDP, potassium dibasic phosphate;
PAS, potassium aluminum sulfate; CS, copper sulfate.

Table III. Incidences of Renal Lesions and Renal Cell Tumors in Nephrectomized Rats Treated with EHEN and Test Chemicals

Group	Treatment	Effective No. of rats	No. of rats with (%)		
			Simple hyperplastic foci	Adenomatous hyperplastic foci	Renal cell tumors
I	EHEN+5%PDP	20	20 (100)[b]	17 (85)[a, b]	6 (30)[b]
II	5%PDP	20	9 (45)[b]	0	0
III	EHEN+5%PAS	20	14 (70)[a, b]	3 (15)	0
IV	5%PAS	20	11 (55)[b]	0	0
V	EHEN+0.5%CS	20	13 (65)[a, b]	3 (15)	0
VI	0.5%CS	20	7 (35)	0	0
VII	EHEN	20	20 (100)[b]	5 (25)	0
VIII	Control	20	2 (10)	0	0

EHEN, N-ethyl-N-hydroxyethylnitrosamine; PDP, potassium dibasic phosphate;
PAS, potassium aluminum sulfate; CS, copper sulfate.
a) Significantly different ($P<0.05$) from group VII.
b) Significantly different ($P<0.05$) from group VIII.

Table IV. Numbers of Renal Lesions and BrdU-labeled Cells in Nephrectomized Rats Treated with EHEN and Test Chemicals

Group	Treatment	Simple hyperplastic foci	Adenomatous hyperplastic foci	Renal cell tumors	BrdU (cells/mm^2)	Nephropathy (Grade)	Mineralization
I	EHEN+5%PDP	6.70±2.45[a]	2.25±1.25[a, b]	0.35±0.58	8.05±1.72[a, b] (4.05±0.87)	3	+
II	5%PDP	0.88±0.95	0	0	7.15±0.38[a, b] (2.90±0.68)	3	+
III	EHEN+5%PAS	1.70±1.26[a]	0.23±0.56	0	6.80±0.53[a, b] (4.13±1.16)	1	−
IV	5%PAS	0.70±0.73	0	0	6.60±1.63[b] (4.55±1.18)	1	−
V	EHEN+0.5%CS	2.00±1.13[a]	0.20±0.41	0	6.90±1.09[b] (4.00±0.86)	1	−
VI	0.5%CS	0.60±0.74	0	0	5.10±0.26[b] (2.85±0.50)	1	−
VII	EHEN	3.20±1.79[b]	0.40±0.57	0	4.73±1.36 (2.73±1.01)	0	−
VIII	Control	0.40±0.55	0	0	3.40±0.86 (2.95±0.79)	0	−

EHEN, N-ethyl-N-hydroxyethylnitrosamine; PDP potassium dibasic phosphate;
PAS, potassium aluminum sulfate; CS, copper sulfate.
( ): Numbers of BrdU-labeled cortical cells without adenomatous hyperplasia and adenoma.
a) Significantly different ($P<0.05$) from group VII.
b) Significantly different ($P<0.05$) from group VIII.

(14/20) in group III, 55% (11/20) in group IV, 65% (13/20) in group V, 35% (7/20) in group VI, 100% (20/20) in group VII and 10% (2/20) in group VIII. Incidences of rats with adenomatous hyperplastic foci were 85% (17/20) in group I, 15% (3/20) in groups III and V, 25% (5/20) in group VII and 0% in groups II, IV, VI and VIII. Incidences of renal cell tumors were 30% (6/20) in group VII and 0% in the other groups.

Mean numbers of simple hyperplastic foci, adenomatous hyperplastic foci and renal cell tumors per animal in each group are summarized in Table IV. The numbers of simple hyperplastic foci per animal in groups I, III and

Fig. 1. Simple hyperplastic foci (arrows), consisting of cells with large nuclei and abundant basophilic cytoplasma of a nephrectomized rat treated with EHEN and PDP (HE stain. ×250).

Fig. 2. Adenomatous hyperplastic foci (small arrows), a renal tubular cell tumor (large arrows) and calcification (arrowhead) of a nephrectomized rat treated with EHEN and PDP are shown (HE stain. ×100).

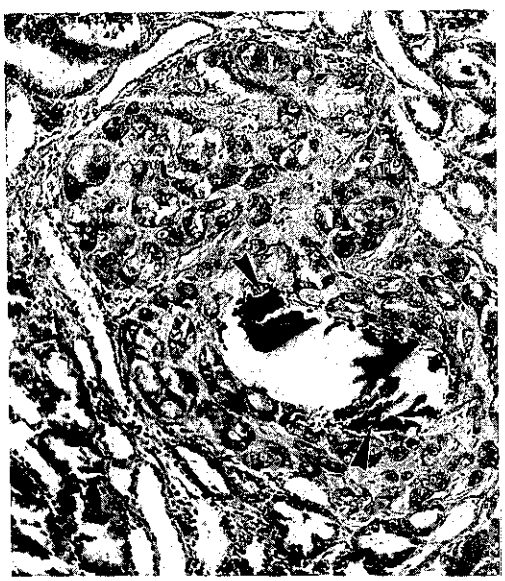

Fig. 3. Calcification (arrowheads) in a renal cell tumor of a nephrectomized rat treated with EHEN and PDP in group I (HE stain. ×250).

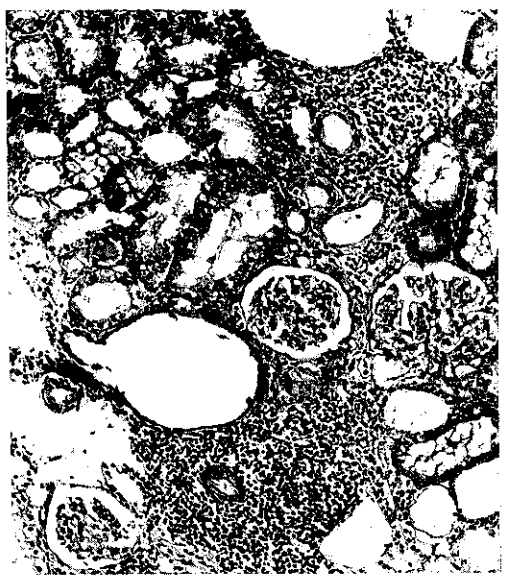

Fig. 4. Lymphocyte accumulation and calcification (arrows) of kidney in a nephrectomized rat treated with EHEN and PDP (HE stain. ×250).

V were significantly higher than that in group VII. The numbers of adenomatous hyperplastic foci per animal in group I alone were significantly higher than that in group VII.

BrdU-labeling indices The mean numbers of BrdU-labeled cells in each group are shown in Table IV. Numbers of BrdU-labeled cells/mm^2 in randomly selected areas containing preneoplastic, neoplastic snd surround-

ing histochemically normal-looking cells in the renal cortex of four sagittal renal sections in groups I, II and III were significantly higher than those in groups VII and VIII. That in group I was the highest. Those in groups I–VI were all significantly higher than that in group VIII. The mean numbers of BrdU-labeled cells in randomly selected cortical areas without preneoplastic and neoplastic lesions were not significantly different in each group.

Other histological findings Findings regarding degree of nephropathy based on the earlier published criteria[13] and calcification in each group are also summarized in Table IV. Nephropathy was seen in groups I–VI. Lymphocyte accumulation, hyaline droplets in the proximal convoluted tubular cells and dilatation of proximal convoluted tubules were seen in the cortex of groups I and II (Fig. 4). Calcification was observed in the renal medulla and cortex of groups I and II (Fig. 4) and in some renal tubular cell tumors (Fig. 3). Slight crystal deposition was also present in proximal convoluted tubular cells in groups III–VI.

DISCUSSION

The present investigation of the potential promoting influence of PDP, PAS and CS on the development of renal tubular cell lesions in nephrectomized rats pretreated with EHEN revealed significant enhancement only for PDP. The efficacy of our medium-term model of bioassay for renal carcinogenesis modifiers has been described previously[11] and the results documented here clearly suggest a link between toxicity-dependent proliferation and promoting ability. In order to enhance proliferation of tubular cells, unilateral nephrectomy was used in the present study, as in previous studies.[14–16]

Early proliferative lesions of tubular epithelial cell origin with slightly basophilic cytoplasm and atypical nuclei, classified as simple hyperplastic foci in the present study, partly correspond to the early neoplastic nodules,[7] atypical cell populations,[17] focal areas of dysplastic tubular epithelium,[18] atypical cell foci[19] and altered tubules[20] of other authors, who gave no clear criteria for size definition. Our adenomatous hyperplastic foci correspond to the microscopic nodules,[20] small nodules[21] or microadenomas[22] described earlier and may be viewed as either preneoplastic or neoplastic, depending on morphological and biological growth characteristics, including lack of a capsule and presence or absence of compressive growth.

In a preliminary experiment of 8 weeks duration, nephrotoxicity of high doses of various salts was examined. The dose of 50,000 ppm PDP increased kidney weight. It was associated with calcification in the medulla and cortex, dilatation of proximal convoluted tubules, hyaline droplets in the proximal convoluted tubular cells and lymphocyte infiltration in the cortex. Hydropic or cloudy swelling, and regeneration of renal proximal convoluted tubular cells were seen in rats treated with 50,000 ppm PDP. The doses of 50,000 ppm PAS and 5,000 ppm CS only induced deposits in the proximal convoluted tubular cells but did not increase kidney weight. It is suggested that hyperplasia induced by 50,000 ppm PDP is more remarkable than those by 50,000 ppm PAS and 5,000 ppm CS.

β-Cyclodextrin,[1] DL-serine,[2] basic lead acetate[3] and trisodium nitrilotriacetate monohydrate[4] were all found to increase kidney weight, induce degeneration of proximal convoluted tubular cells and promote the development of renal tubular cell tumors in unilaterally nephrectomized rats treated with EHEN. They did not cause calcification. Diethylene glycol, while being a nephrotoxin, does not promote the development of renal tubular cell tumors in rats treated with EHEN and subjected to nephrectomy. It also did not significantly increase kidney weight or the number of BrdU-labeled cells.[11, 13] This finding, taken together with the present results, clearly suggests that nephrotoxic substances exert promoting potential in line with their ability significantly to increase the kidney weight or number of BrdU-labeled cells.

There have been many reports concerning the promoting effect of urinary bladder stones on the development of bladder tumors[23–27] and renal cell tumors associated with renal calcification.[28–30] In the present experiment, the calcification induced by PDP or nephropathy associated with the calcification might similarly have provided the stimulus to enhance development of renal cell tumors in rats treated with EHEN.

In conclusion, the present studies revealed that 50,000 ppm PDP alone increases kidney weight and significantly promotes the development of renal tubular cell tumors. On the other hand, 50,000 ppm PAS and 5,000 ppm CS exerted no promoting effect and did not increase kidney weight.

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