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Promoting Effect of the Peroxisome Proliferator, Clofibrate, but Not Di(2-ethylhexyl)phthalate, on Urinary Bladder Carcinogenesis in F344 Rats Initiated by N-Butyl-N-(4-hydroxybutyl)nitrosamine

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The modifying potential of clofibrate and di(2-ethylhexyl)phthalate (DEHP) on second stage, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-initiated urinary bladder carcinogenesis was investigated in male F344 rats, using a uracil-accelerated transitional cell proliferation model. Six-week-old animals received 0.05% BBN in their drinking water for 4 weeks and then clofibrate (1.0, 0.5, and 0.25%) and DEHP (1.2, 0.6, and 0.3%) were given during experimental weeks 5–8 and weeks 12–20. Uracil was administered during weeks 9–11 at a dietary level of 3.0%. Control rats were treated with BBN and uracil without peroxisome proliferator. Surviving animals were killed at the end of week 20 of the experiment, when the densities of putative preneoplastic, papillary or nodular (PN) hyperplasias (numbers per 10 cm of basement membrane) were significantly increased in all clofibrate-treated, but not the DEHP groups. The incidences of PN hyperplasia were similar in both treated animals and controls. In a second experiment, rats fed diets containing 1.0% clofibrate or 1.2% DEHP were assessed for levels of DNA synthesis in urinary bladder epithelium by 5-bromo-2′-deoxyuridine immunohistochemistry. Numbers of labeled nuclei remained within normal levels, and no proliferative changes were evident. Thus, the present experiments indicated that while clofibrate, but not DEHP, exerts weak enhancing effects on BBN-initiated urinary bladder carcinogenesis in rats this is not associated with increased levels of DNA synthesis in the affected epithelium.

Key words: Promoting effect — Peroxisome proliferator — Clofibrate — Di(2-ethylhexyl)phthalate — Urinary bladder carcinogenesis

Peroxisome proliferators, which comprise two structurally dissimilar groups of chemicals, namely the hypolipidemic drugs and certain phthalate ester plasticizers, form a novel class of non-genotoxic hepatocarcinogens.[1,2] These chemicals have shown equivocal results in liver tumor promotion studies.[3–9] Although the precise mechanisms underlying hepatocarcinogenesis by peroxisome proliferators remain unclear, it is considered most likely that high β-oxidation enzyme activity, leakage of H_2O_2 from peroxisomes and subsequent oxidative hepatocyte[10,11] or DNA[12,13] damage and replicative DNA synthesis[14,15] are involved. Given the suspected wide role played by oxidative damage in carcinogenesis, peroxisome proliferators might be expected to exert effects in many organs.

Clofibrate was found not to modify the carcinogenic potential of N-methyl-N-nitrosourea or dimethylhydrazine, however, and in fact inhibited mammary carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene.[16] In contrast, a recent investigation in our laboratory demonstrated that clofibrate can increase the induction of rat urinary bladder transitional cell hyperplasia and papillomas in a wide-spectrum organ carcinogenesis model.[17] Diwan *et al.*[18] reported that DEHP[2] possesses enhancing effects on transformation to anchorage-independent growth by JB6 mouse epidermal cells, and also promotes second-stage skin tumor development in mice. Furthermore, Kurokawa *et al.*[19] have demonstrated that DEHP also acts as a promoter of 2-stage rat renal carcinogenesis.

The objective of the present studies was to assess and compare the modifying effects of typical representatives of the two types of peroxisome proliferator, clofibrate and DEHP, on urinary bladder carcinogenesis, using an early prediction model including uracil as an accelerator of transitional cell proliferation,[20] in rats initiated with BBN. An investigation of their effects on levels of DNA synthesis in urinary bladder epithelium after 8 weeks of exposure was also included.

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[2] Abbreviations: DEHP, di(2-ethylhexyl)phthalate; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BrdU, 5-bromo-2′-deoxyuridine.

MATERIALS AND METHODS

Animals Male F344/DuCrj rats, 5 weeks of age, were obtained from Charles River Japan, Inc., Kanagawa. They were housed five to a plastic cage with hardwood chips for bedding, and fed MF powdered diet (Oriental Yeast, Co., Ltd., Tokyo) and water *ad libitum*. Animals were maintained at a temperature of 22±2°C and humidity of 55±10% with a 12-h light/dark cycle. After a one-week acclimation period, they were used in this investigation.

Chemicals BBN, clofibrate and DEHP were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Uracil was obtained from Yamasa Shoyu Co., Choshi.

Initiation-promotion study (experiment 1) A total of 125 rats were divided randomly into 3 groups, groups 1 and 3 being subdivided into subgroups for treatment with test chemicals at three different dietary levels. Rats in group 1 were given drinking water with 0.05% BBN for 4 weeks and then were maintained on diets containing clofibrate at 1.0, 0.5 and 0.25% (groups 1a–1c), or DEHP at 1.2, 0.6, and 0.3% (groups 1d–1f) and also fed diet containing 3.0% uracil for the experimental durations as shown in Fig. 1. Group 2 was treated with BBN and uracil, but not test chemicals, and served as a control. Group 3 animals were maintained on diets containing clofibrate at 1.0% (group 3a) or DEHP at 1.2% (group 3d), and diet containing uracil at 3.0% as in group 1 without prior carcinogen exposure.

The animals were observed daily for abnormalities and were weighed every week for the first 6 weeks, and then at two-week intervals. Food and water consumptions were measured over a 2-day period before each time of weighing. During week 20, before termination, fresh urine samples were obtained from at least five rats in each group. The pH of the samples was measured with a pH meter (Hitachi-Horiba Co., Kyoto). Urine samples collected over a 4 h period were centrifuged and the precipitates, stained with Sternhimer & Malbin solution (Muto Pure Chemicals Ltd., Tokyo), were examined under light microscopy for epithelial cells, red blood cells, white blood cells, crystals, and casts.

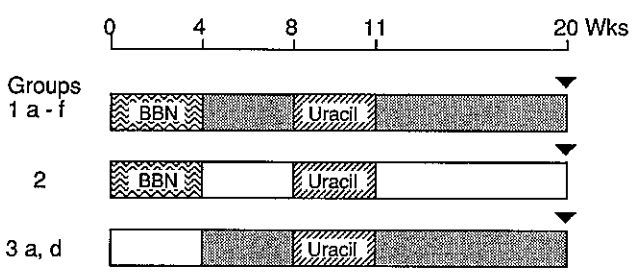

Fig. 1. Experimental design: 0.05% BBN (▨) in the drinking water; 3.0% uracil (▨) in the diet; test chemical (▨) in the diet, a–c: 1.0, 0.5 and 0.25% clofibrate, d–f: 1.2, 0.6 and 0.3% DEHP; basal diet (□); termination (▼).

At week 20, all animals were deprived of food, but not water, overnight and then killed under ether anesthesia by exsanguination (blood samples were collected) from the aorta. After separation, the sera were analyzed for glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, triglyceride, phospholipid, and free fatty acid. The spleens, livers, kidneys and testes were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. The urinary bladders were fixed by inflation with and immersion in the same fixative, halved and examined under a dissecting microscope. They were then cut into 8 strips, embedded in paraffin, sectioned, stained with hematoxylin and eosin and examined histologically. For quantitative analysis, urinary bladder lesions were counted under the light microscope, the total length of the basement membrane being measured with a color video image processor (SPICCA-II; Nippon Avionics Co., Ltd., Tokyo), and numbers of lesions per 10 cm of basement membrane were recorded. The spleens, livers, kidneys and testes were also examined histologically.

Eight-week toxicity study (experiment 2) Eighteen male rats were randomly divided into three equal groups. They were given diets containing 1.0% clofibrate, 1.2% DEHP or basal diet and weighed weekly. After week 8 all rats were killed 1 h after an ip injection of BrdU (Sigma Chemical Co., St. Louis, MO) at a dose of 100 mg/kg body weight. The final body, liver and kidney weights were measured, and organ-to-body weight ratios were determined. Liver, kidney, urinary bladder, and small intestine tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were incubated in a 1:2000 dilution of mouse monoclonal antibody to BrdU (Becton Dickinson, Mountain View, CA) after pretreating the slides with 4 *N* HCl for 20 min at 37°C and rinsing in boric acid-borate buffer, pH 7.6, then 0.02% actinase (Kaken Seiyaku Co., Ltd., Tokyo), and stained by the ABC method using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). These slides were lightly counterstained with hematoxylin. The numbers of labeled nuclei per 1000 renal tubular or hepatocyte nuclei were counted. For urinary bladder, the numbers of labeled epithelial cells were counted per centimeter of epithelium.

Statistical analysis The significances of intergroup differences in numerical data obtained for final body weights, organ weights, and quantitative histopathological findings were assessed using the two-sided Student's *t* test. Insufficient homogeneity of variance was corrected with respect to the degrees of freedom according to Welch.

RESULTS

Experiment 1

Survival, body weights, food and water consumption data No clinical abnormalities or mortalities related to the test peroxisome proliferator administration were apparent in any of the groups during the experimental period. Body weight gain was slightly to markedly (dose-dependent manner) retarded in rats treated with clofibrate or DEHP(groups 1a–1f). This tendency was also observed in groups 3a and 3d treated with clofibrate or DEHP without BBN. The average BBN and uracil intakes in groups 1a–1f and 2 were 52–55 mg/kg/day and 1213–1329 mg/kg/day, respectively, no significant intergroup differences being apparent. Rats in group 1a given 1.0% clofibrate consumed less food than the controls, and no remarkable changes were found in groups 1b–1f. The group 1a animals also drank less water than the controls from week 5 to the end of the experiment.

Urinalysis and blood biochemistry data Results of urinalysis of samples taken at week 20 did not reveal any test chemical treatment-related changes (data not shown). In blood biochemistry, reduction of triglyceride and free fatty acid values, and elevated alkalinephosphatase levels were apparent in rats receiving clofibrate or DEHP with clear dose dependency (Table I). No treatment-related changes were found in any of the other parameters examined.

Organ-to-body weight ratios The liver-to-body weight ratios in groups 1a–1f that received clofibrate or DEHP, and especially at high dietary levels, were significantly greater than control values (Table II). The kidneys-to-body weight ratios in groups 1a–1f, and the testes-to-body weight ratios in groups 1a–1d were also significantly increased as compared to control values. However, these changes were considered to be related to the observed body weight retardation.

Table I. Experiment 1 Blood Biochemistry Data

Group and treatment[a]		No. of rats	Alkaline phosphatase (K-A)	Triglyceride (mg/dl)	Phospholipid (mg/dl)	Free fatty acid (meq/liter)
1a	BBN; 1.0% Clofibrate	15	24.95±1.62**	31.3±4.2**	96.5±7.1	0.495±0.102**
b	BBN; 0.5% Clofibrate	15	19.51±1.26**	37.0±10.7**	99.8±5.6	0.589±0.089**
c	BBN; 0.25% Clofibrate	15	13.65±1.13**	59.7±13.8**	104.3±9.0*	0.689±0.081**
d	BBN; 1.2% DEHP	15	15.74±0.66**	45.7±8.8**	105.1±4.5*	0.672±0.068**
e	BBN; 0.6% DEHP	15	13.77±0.98**	51.2±11.7**	104.7±6.7	0.735±0.070**
f	BBN; 0.3% DEHP	15	11.47±0.58**	60.7±13.8**	104.3±10.1	0.701±0.075**
2	BBN	15	9.78±0.73	97.4±22.0	90.7±24.4	0.807±0.067

*, ** Statistically significant as compared to group 2 values ($P<0.05$ or 0.01).
a) Data for groups 3a and 3d were excluded from this table, since BBN treatment did not affect any of these parameters.
Data are mean±SD values.

Table II. Experiment 1 Final Body Weights and Organ-to-Body Weight Ratios

Group and treatment[a]		No. of rats	Final body weight (g)	Liver (%)	Kidneys (%)	Testes (%)
1a	BBN; 1.0% Clofibrate	15	233±18**	4.64±0.21**	0.96±0.05**	1.22±0.04**
b	BBN; 0.5% Clofibrate	15	310±18**	3.91±0.20**	0.87±0.06**	0.98±0.05**
c	BBN; 0.25% Clofibrate	15	332±13**	3.13±0.17**	0.79±0.04**	0.92±0.03**
d	BBN; 1.2% DEHP	15	332±15**	3.99±0.16**	0.75±0.03**	0.94±0.05**
e	BBN; 0.6% DEHP	15	347±14*	3.30±0.12**	0.70±0.05**	0.87±0.06
f	BBN; 0.3% DEHP	15	352±15	2.74±0.10**	0.63±0.03**	0.86±0.04
2	BBN	15	359±17	2.27±0.07	0.60±0.02	0.85±0.03

*, ** Statistically significant as compared to group 2 values ($P<0.05$ or 0.01).
a) Data for groups 3a and 3d were excluded from this table, since BBN treatment did not affect any of these parameters.
Data are mean±SD values.

Table III. Experiment 1 Histopathological Findings in the Urinary Bladder

Group and treatment		No. of rats	PN hyperplasia		Papilloma	
			Cases (%)	Density[a]	Cases (%)	Density
1a	BBN; 1.0% Clofibrate	15	15 (100.0)	12.14±6.04**	8 (53.3)	1.19±1.86
b	BBN; 0.5% Clofibrate	15	15 (100.0)	11.32±5.16**	12 (80.0)	1.89±1.47
c	BBN; 0.25% Clofibrate	15	15 (100.0)	10.49±3.33**	12 (80.0)	1.23±1.08
d	BBN; 1.2% DEHP	15	15 (100.0)	6.09±4.17	10 (66.7)	1.11±0.97
e	BBN; 0.6% DEHP	15	13 (86.7)	5.39±3.83	4 (26.7)	0.39±0.70
f	BBN; 0.3% DEHP	15	15 (100.0)	7.08±3.47*	11 (73.3)	0.82±0.71
2	BBN	15	15 (100.0)	4.85±1.85	10 (66.7)	1.10±1.09
3a	1.0% Clofibrate	10	0		0	
d	1.2% DEHP	10	0		0	

*, ** Statistically significant as compared to group 2 values ($P<0.05$ or 0.01).
a) Density is expressed as the number of lesions per 10 cm of basement membrane. Data are mean±SD values.

Table IV. Experiment 2 Final Body Weights and Organ-to-Body Weight Ratios

Group and treatment		No. of rats	Final body weight (g)	Liver (%)	Kidneys (%)
1	1.0% Clofibrate	6	166±12**	5.71±0.27**	0.93±0.05**
2	1.2% DEHP	6	272±10**	4.43±0.13**	0.76±0.05**
3	Control	6	298±7	2.59±0.05	0.62±0.02

** Statistically significant as compared to group 3 values ($P<0.01$).
Data are mean±SD values.

Table V. Experiment 2 BrdU Immunohistochemistry: Nuclear Labeling Indices in Urinary Bladder Epithelium, Cortical Renal Tubules and Hepatocytes

Group and treatment		No. of rats	Labeling index[a]		
			Urinary bladder epithelium	Cortical renal tubules	Hepatocytes
1	1.0% Clofibrate	6	2.63±2.35	1.33±1.21	1.00±0.89
2	1.2% DEHP	6	0.33±0.52	1.33±1.21	0.00±0.00
3	Control	6	0.74±0.66	1.67±1.63	0.00±0.00

a) For urinary bladder, number of labeled nuclei/cm of urothelium. For kidneys and liver, number of labeled renal cortical tubular cell or hepatocyte nuclei/1000 cells counted. Data are mean±SD values.

Incidences and densities of urinary bladder proliferative lesions Quantitative data regarding urinary bladder proliferative lesions are summarized in Table III. The lesions observed in the urinary bladder mucosae were classified as simple hyperplasia, PN hyperplasia, papilloma and carcinoma on the basis of the criteria described previously.[21] Simple hyperplasia was observed in all rats initiated with BBN, and was excluded from the quantitative analysis. No significant increases in incidence of PN hyperplasia were apparent in groups 1a–1f when compared to the controls (group 2). Quantitative analysis, however, revealed significantly increased densities of PN hyperplasia in groups 1a–1c, receiving clofibrate, but not in rats given DEHP (groups 1d–1f). There were no significant differences in the incidences of papillomas between control and experimental groups. Rats given peroxisome proliferators and uracil without BBN (groups 3a and 3d) developed mild simple hyperplasia,

but did not demonstrate any PN hyperplasias and papillomas.

Pathology of other organs Eosinophilia of hepatocytes occurred in all rats exposed to clofibrate and DEHP (groups 1a–1f and 3a, 3d), this lesion not being observed in group 2. No treatment-related changes were observed in the other organs examined.

Experiment 2

The mean body weights in the 1.0% clofibrate and 1.2% DEHP groups were significantly lower than those of the control group from weeks 1 to 8. The liver- and kidneys-to-body weight ratios were significantly increased when compared to the control values (Table IV).

As determined by BrdU immunohistochemistry, while the nuclear labeling index (LI) of the 1.0% clofibrate group urinary bladder epithelium tended to increase, this was not statistically significant (Table V). Histopathologically, no proliferative urinary bladder lesions were observed. LI values of renal cortical tubules and hepatocytes were not affected by clofibrate or DEHP treatment.

DISCUSSION

It has been reported that the tumor-promoting activity of clofibrate, a non-genotoxic hepatocarcinogen, is limited to the liver.[16] However, we clearly demonstrated that clofibrate can enhance the induction of neoplastic proliferative lesions in rat urinary bladder epithelium with a wide-spectrum organ carcinogenesis model, using a combination of three initiating carcinogens, namely diethylnitrosamine, N-methylnitrosourea and dihydroxydi-N-propylnitrosamine.[17] Thus, in view of this urinary bladder tumor-promoting potential, there is a need to evaluate the effects of other peroxisome proliferators. The present BBN initiation-promotion model, using uracil to induce transitional cell proliferation, has proved of advantage for this task, confirming an enhancing effect for clofibrate. Thus, the average numbers of PN hyperplasias per 10 cm of basement membrane were significantly increased in all groups exposed to this agent following BBN and uracil treatment (Table III). The reason for the lack of any clear dose-dependency is unclear, but possibly a plateau effect was reached at a very low level. However, no such influence was observed for DEHP, another type of peroxisome proliferator which is also a non-genotoxic hepatocarcinogen, despite its reported promoting effects on skin[18] and renal carcinogenesis.[19]

Rats exposed to both clofibrate or DEHP suffer similar peroxisome proliferator-specific changes, including reduction of triglyceride and free fatty acid levels in the serum, increased liver weight and eosinophilia of hepatocytes. From this evidence, it may be concluded that the promoting effect of clofibrate for rat urinary bladder carcinogenesis is not directly related to peroxisome proliferation.

Whereas the precise mechanisms underlying promotion of urinary bladder carcinogenesis remain unclear, other known promoters, such as sodium saccharin, antioxidants and sodium salts induce urothelial hyperplasia, and increase DNA synthesis.[22,23] However, in the present study, neither induction of hyperplastic lesions nor increase in urinary bladder epithelium DNA synthesis were observed in rats fed 1.0% clofibrate and 1.2% DEHP for 8 weeks. Similar results were also obtained for another bladder tumor promoter, sodium barbital,[24] suggesting that additional factors are involved in the bladder carcinogenesis-promoting effects of certain types of chemicals. More information concerning this point awaits the development of appropriate models.

To evaluate the modifying effects of chemicals in urinary bladder carcinogenesis, we have developed a system involving treatment with BBN for 4 weeks followed by continuous administration of test chemicals for 32 weeks.[25,26] With this model, it was established that post-treatment with several sodium or potassium salts, antioxidants, urolithiasis-inducing chemicals, anticancer agents and amino acids exerts enhancing effects on urinary bladder carcinogenesis.[25-27] Recently, we have succeeded in shortening the experimental period of the aforementioned model,[20] by applying uracil in the middle of the post-initiation stage to induce reversible transitional cell proliferation[28-30] and to increase DNA synthesis transiently.[31] Further application of this approach should allow relatively rapid assessment of the effects of large numbers of compounds, which should in turn cast light on possible mechanisms.

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