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Parp-1 deficiency does not increase the frequency of tumors in the oral cavity and esophagus of ICR/129Sv mice by 4-nitroquinoline 1-oxide, a carcinogen producing bulky adducts

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Abstract

The impact of poly(ADP-ribose) polymerase-1 (*Parp−1*)-deficiency on 4-nitroquinoline 1-oxide (4NQO)-induced carcinogenesis was studied in mice with an ICR/129Sv mixed genetic background. *Parp-1*$^{+/+}$, *Parp−1*$^{+/-}$ and *Parp-1*$^{-/-}$ animals given 4NQO for thirty-two weeks at 0.001% in their drinking water developed papillomas and squamous cell carcinomas of the tongue, palate and esophagus, but with no statistically significant variation with the *Parp−1* genotype. Thus *Parp-1* deficiency does not elevate susceptibility to carcinogenesis induced by a carcinogen which gives rise to bulky DNA lesions. This study also indicated that the ICR/129Sv mixed genetic background is associated with high yield induction of esophageal tumors by 4NQO.

Keywords: 4-Nitroquinoline 1-oxide; Poly(ADP-ribose) polymerase-1; Squamous cell carcinoma; Carcinogenesis; Knockout mice

1. Introduction

Poly(ADP-ribose) polymerase (Parp)-1 is activated by DNA strand breaks and polyADP-ribosylates various nuclear proteins, including itself and histones, using NAD as a substrate [1,2]. Accumulated evidence indicates that Parp−1 is involved in base excision repair (BER) as well as repair of single- and double-strand breaks [3–6] and *Parp-1*$^{-/-}$ mice are generally more susceptible than their *Parp−1*$^{+/+}$ counterparts to carcinogenic activity of alkylating agents [7,8]. Incidences of tumors have also been found to be augmented in SCID*Parp-1*$^{-/-}$ [5] and *Ku80*$^{+/-}$ *Parp−1*$^{-/-}$ [9] as compared with *Parp-1*$^{+/+}$ mice, in good accordance with the accepted role of Parp−1 in BER and DNA strand break repair.

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While, it is postulated that Parp-1 does not contribute to nucleotide excision repair (NER) [10], the main pathway for repair of bulky DNA lesions, its involvement in cell death accompanying NAD depletion [6,11,12] and also in the maintenance of genomic stability [13–15] as well as control of differentiation [16–18], suggest that *Parp*$-1^{-/-}$ mice might be susceptible to carcinogenesis by all types of carcinogens, independent of the type of DNA adducts that they generate. However, in contrast to the high susceptibility to carcinogenesis induced by alkylating agents in *Parp-1*$^{-/-}$ mice, tumor yields in response to a heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), which produces bulky DNA adducts, were not elevated in the lungs and liver [19]. To further clarify the impact of *Parp*-1 deficiency on carcinogenesis induced by different types of carcinogens, we here employed 4-nitroquinoline 1-oxide (4NQO) [20], which give rises to DNA adducts that may be mainly repaired through NER like UV-induced DNA lesions [21], in a mice strain with a mixed genetic background of 129Sv/ICR.

2. Materials and methods

2.1. Mice

Parp-1$^{+/-}$ and *Parp*$-1^{-/-}$ mice used in this study were generated by disrupting the *Parp-1* exon 1 through the insertion of a neomycin resistance gene cassette as described previously [22]. *Parp*$-1^{+/+}$, *Parp*$-1^{+/-}$ and *Parp*$-1^{-/-}$ male mice with a mixed genetic background of ICR/129Sv were produced by brother–sister mating of *Parp*$-1^{+/-}$ mice [22]. Genotypes were determined by Southern blot analysis using tail-tip DNA samples as described elsewhere [22]. The animals were housed in plastic cages in an air-conditioned room with a 12 h light–dark cycle and basal diet (CE-2, CLEA JAPAN, Tokyo, Japan) and sterilized water were available *ad libitum*. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of the participating institutions.

2.2. Carcinogenesis experiment

4NQO (Sigma) was dissolved and diluted with sterilized drinking water to achieve a concentration of 0.001% and administered orally *ad libitum* from light-shielded polyvinyl bottles for 32 weeks, starting at the age of 9 weeks. The bottles were changed with fresh 4NQO solution once a week and consumption was recorded to estimate the intake of 4NQO. Five each of the *Parp*$-1^{+/+}$, *Parp*$-1^{+/-}$ and *Parp*$-1^{-/-}$ mice were used as controls and fifteen *Parp*$-1^{+/+}$, eighteen *Parp*$-1^{+/-}$ and eleven *Parp*$-1^{-/-}$ mice served for 4NQO treatment. Body weights of the mice were measured once a week. At 32 weeks after the initiation of 4NQO administration, mice were anesthetized with ether and autopsies were performed. All organs were examined macroscopically for the presence of tumors and the tongue, esophagus and stomach were fixed in neutralized 10% formalin solution and routinely processed for embedding in paraffin. For the palate and nasal cavity, decalcification was performed with formic acid/formalin fixation. Sections were cut at 3 μm thickness and stained with hematoxylin and eosin (HE) for histopathological analysis.

2.3. Statistical analysis

The χ^2 analysis and Fisher's exact tests were performed to compare data for incidences and the Student-*t* test and Wilcoxon-Mann-Whitney-U test for the body weights, 4NQO intake and multiplicities of the tumors, using SSPS software on Macintosh computers.

3. Results

Three *Parp*$-1^{-/-}$ mice demonstrated loss of condition and were subjected to autopsy at 29 or 31 weeks after initiation of 4NQO administration, all harbouring tumors in either the oral cavity or the esophagus. The experiment was therefore terminated at 32 weeks and autopsy was performed for the remaining animals. There were no significant differences in the initial mean body weights among the genotypes (Table 1). At the end of experiment, an apparent increase of body weights was observed in *Parp*$-1^{+/+}$ and *Parp*$-1^{+/-}$ mice of the 4-NQO-treated ($P=0.76$ and $P<0.005$, respectively) and untreated groups ($P=0.008$ and $P=0.007$, respectively) but not in *Parp*$-1^{-/-}$ mice. The values for *Parp*$-1^{+/-}$ mice were slightly higher than those for *Parp*$-1^{-/-}$ mice in both 4-NQO-treated and untreated groups ($P=0.034$ and $P=0.008$, respectively), but the values for 4-NQO-treated *Parp*$-1^{+/+}$ and *Parp*$-1^{-/-}$ mice were not statistically different. Total intake of 4NQO did not significantly differ among the *Parp*-1 genotypes (data not shown).

As summarized in Tables 2 and 3, tumors were observed in the oral cavity, esophagus and stomach after 4NQO administration in animals of each genotype, but not in the control groups. One *Parp*$-1^{+/-}$ mouse given

Table 1
Initial and final body weights

Group	*Parp-1*	4NQO (0.001%)	Effective no. of mice	Initial body weight (g)[a]	Final body weight (g)[a]
1	+/+	+	15	31.0±0.7	34.5±1.7
2	+/−	+	18	31.7±0.9	36.8±1.9[b]
3	−/−	+	11	31.2±0.9	33.7±1.3[c, d]
4	+/+	−	5	29.6±0.5	37.1±1.2[e]
5	+/−	−	5	32.3±1.0	42.5±1.5[d, e]
6	−/−	−	5	32.9±2.3	33.6±0.9[f]

[a] Mean + SE.
[b] Significantly different from the initial body weight (at $P < 0.0001$).
[c] Three mice were subjected to autopsy before the termination of experiment and mean body weight of the remaining mice at thirty-two weeks was measured.
[d] Significantly different from the final body weight of the *Parp*$-1^{+/-}$ group given 4NQO (at $p < 0.05$).
[e] Significantly different from the initial body weight (at $P < 0.01$).
[f] Significantly different from the final body weight of the untreated *Parp*$-1^{+/-}$ group (at $P < 0.01$).

4NQO developed a tumor in the Harderian glands. Tumors in the oral cavity were mainly observed in the tongue, as in *XPA*$^{-/-}$ mice with the C57BL/6 genetic background [23], but also in the palate but not the nasal cavity. There were no significant differences in the incidences, multiplicities or histology of hyperplasia, papillomas and squamous cell carcinomas (SCCs) in the oral cavity among the *Parp*-1 genotypes.

High incidences of hyperplasia and papillomas, and to a lesser extent SCCs, were also observed in the esophagus of *Parp*$-1^{+/+}$ mice as well as other genotypes, again with no statistically significant influence of the *Parp*-1 genotype. In the stomach, two *Parp*$-1^{+/+}$ mice harboured a papilloma and an SCC and hyperplasia was observed in one *Parp*$-1^{-/-}$ mouse.

4. Discussion

The present study demonstrated no variation in susceptibility to 4NQO carcinogenicity among *Parp*$-1^{+/+}$, *Parp*$-1^{+/-}$ and *Parp*$-1^{-/-}$ mice. Because the incidences of papillomas and SCCs in the oral cavity and of SCCs in the esophagus were relatively low, the absence of any differences in the incidences was not due to a saturation dose of 4NQO. We also recently found that the susceptibility to carcinogenesis induced by a heterocyclic amine, IQ, did not differ between *Parp*$-1^{+/+}$ and *Parp*$-1^{-/-}$ mice with a C57BL/6 genetic background [19]. It is known that genetic background can affect the outcome of carcinognenesis experiments but our previous results showed increased susceptibility of the same

Table 2
Incidences and multiplicities of tumors in the oral cavity

	Parp-1	4NQO (0.001%)	Effective no.of mice	Tongue			Palate		
				Hyperplasia	Papilloma	SCC[a]	Hyperplasia	Papilloma	SCC[a]
Incidences[b]									
Control	+/+	(−)	5	0	0	0	0	0	0
	+/−	(−)	5	0	0	0	0	0	0
	−/−	(−)	5	0	0	0	0	0	0
4NQO	+/+	(+)	15	9 (56)	5 (33)	3 (20)	6 (40)	1 (6)	0
	+/−	(+)	18	6 (33)	4 (22)	1 (6)	11 (61)	1 (5)	0
	−/−	(+)	11	6 (55)	2 (18)	1 (9)	7 (63)	2 (18)	2 (18)
Multiplicity[c]									
4NQO	+/+	(+)	15	1.3±0.2	1.4±0.3	1.0	1.3±0.2	2.0	0
	+/−	(+)	18	1.3±0.2	1.3±0.3	1.0	1.6±0.2	1.0	0
	−/−	(+)	11	1.3±0.2	1.0±0	1.0	1.6±0.3	1.0	1.0

[a] Squamous cell carcinoma.
[b] No. of mice bearing tumor (%).
[c] No. of tumors per mouse bearing tumor. Mean (when no. of tumors was one) or mean ± SE (when no. of tumors was greater than two).

Table 3
Incidences and multiplicities of tumors in the esophagus and stomach

	Parp-1	4NQO (0.001%)	Effective no.of mice	Esophagus			Stomach		
				Hyperplasia	Papilloma	SCC[a]	Hyperplasia	Papilloma	SCC[a]
Incidences[b]									
Control	+/+	(−)	5	0	0	0	0	0	0
	+/−	(−)	5	0	0	0	0	0	0
	−/−	(−)	5	0	0	0	0	0	0
4NQO	+/+	(+)	15	11 (73)	9 (60)	2 (13)	0	1 (7)	1 (7)
	+/−	(+)	18	13 (72)	13 (72)	6 (33)	0	0	0
	−/−	(+)	11	8 (73)	9 (82)	1 (9)	1 (9)	0	0
Multiplicity[c]									
	+/+	(+)	15	3.9±0.7	4.7±0.9	1.0	0	1.0	1.0
	+/−	(+)	18	3.4±0.5	3.8±0.9	1.3±0.2	0	0	0
	−/−	(+)	11	3.9±0.4	3.6±0.9	2.0	1.0	0	0

[a] Squamous cell carcinoma.
[b] No. of mice bearing tumor (%).
[c] No. of tumors per mouse bearing tumor. Mean (when no. of tumors was two) or mean ± SE (when no. of tumors was greater than two).

Parp$-1^{-/-}$ 129Sv/ICR mice to carcinogenesis induced by alkylating agents [7,8]. The data therefore strongly support our current view that the suppressive role of Parp-1 is limited to particular types of carcinogens, dependent on the repair pathway for individual carcinogen-induced lesions.

4NQO is reduced by nitroreductase [24] or DT diaphorase [25] to a proximate carcinogen, 4-hydroxyquinoline-1-oxide (4HQO), which binds to serine residues in aminoacyl-tRNA synthetase to generate the ultimate carcinogen [26] producing adducts on the N-2 and C-8 positions of guanine residues as well as the N-6 position of adenine residues [27]. 4NQO induces mutations in the bone marrow, lungs and livers as well as in the stomach when administered by gavage [28]. Transversion mutations at guanine residues parallel the amounts of C-8 adducts on guanine residues [29] as well as transition mutations at guanine residues [30], these being mainly repaired by NER like DNA adducts induced by IQ [21]. In the NER pathway, global genome repair, transcription-coupled repair (TCR) and translesion synthesis are known as the major components. Eighty-six and zero percent of *XPA*$^{-/-}$ and *XPA*$^{+/+}$ mice, respectively, were found to develop tongue tumors after administration of 0.001% 4NQO in drinking water for 50 weeks, implying that global genome repair or TCR involving the XPA protein may be important for the removal of lesions induced by 4NQO. In contrast, DNA lesions induced by alkylating agents, such as BHP and azoxymethane, are mainly repaired by base-excision repair (BER), alkylguanine alkyltransferases, DNA strand break repair or homologous recombination repairs (HRR). Therefore, our results support the notion that Parp-1 plays a role in the suppression of carcinogenesis due to alkylation-induced DNA damage, but not through the NER pathway.

4NQO also increases the frequency of chromosomal aberrations [31] and micronucleus formation [32], suggesting that DNA strand breaks could also be induced through oxidative stress [33]. We here terminated the experiment at 32 weeks from the initiation of 4NQO administration because animals bearing tumors were observed from 29 weeks. On the other hand, there are significant differences in the final body weights among the genotypes. *Parp-1*$^{-/-}$ mice of both untreated and 4NQO treated groups did not show gain of body weight, in contrast to *Parp-1*$^{+/+}$ and *Parp-1*$^{+/-}$ mice. 4NQO administration reduced the gain of body weight in *Parp-1*$^{+/-}$ mice, whereas those of *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice were apparently unaffected by 4NQO. The results suggest that 4NQO toxicity might be possibly slightly different among the three genotypes. Therefore, it may be of interest to carry out a longer term experiment using a lower dose of 4NQO to further evaluate the susceptibility to carcinogenesis in *Parp-1*-deficient mice.

Certain nitrosamines, such as benzylmethylnitrosamine and phenylmethylnitrosamine, are known to induce high yields of esophageal tumors in rodents [34]. Steidler et al. reported that when 4NQO was applied repeatedly to the palates of male CBA mice, only 20% developed papillomas or SCCs in the esophagus after 12–16 weeks treatment [35]. We unexpectedly found that our mice with an ICR/129SV mixed genetic background are highly susceptible to

esophageal tumor development induced by 4NQO. The same concentration of 0.001% 4NQO in drinking water administered to the C57BL/6 strain did not result in the generation of esophageal tumors as previously reported [23]. Therefore, the ICR/129SV mouse strain may be particularly useful for studies of the carcinogenic actions of 4NQO.

In conclusion, we demonstrated here that the susceptibility to 4NQO-induced carcinogenesis in the oral cavity and esophagus is not altered by *Parp−1* deficiency.

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Effect of Running Training on DMH-Induced Aberrant Crypt Foci in Rat Colon

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ABSTRACT

FUKU, N., M. OCHIAI, S. TERADA, E. FUJIMOTO, H. NAKAGAMA, and I. TABATA. Effect of Running Training on DMH-Induced Aberrant Crypt Foci in Rat Colon. *Med. Sci. Sports Exerc.*,Vol. 39, No. 1, pp. 70–74, 2007. **Purpose:** We examined the effects of treadmill-running training on the induction of aberrant crypt foci (ACF), which is the first step of colon cancer induction, in the colonic mucosa of rats injected with 1,2-dimethylhydrazine (DMH). **Methods:** Four-week-old F344 rats (N = 38) were randomly assigned to training (19 rats) and control (19 rats) groups. After a week, all rats were given DMH (20 $mg \cdot kg^{-1}$ body weight) once a week for 2 wk. Running training was started at age 7 wk (speed: 10 $m \cdot min^{-1}$, 0% grade, 120 $min \cdot d^{-1}$, 5 $d \cdot wk^{-1}$). After 4 wk of training, the rats were sacrificed and the colon was removed, opened, and counted for ACF with 0.2% methylene blue staining. **Results:** Running training resulted in lower body- ($P < 0.01$) and adipose fat weight ($P < 0.05$). The numbers of ACF and total AC were significantly lower in the running training group than in the control group ($P < 0.05$). The occurrences of one, three, and five aberrant crypts per focus were also significantly lower in the running training group than in the control group ($P < 0.05$). The ratios of total AC/ACF did not significantly differ between the running training and control groups. **Conclusions:** The results of the present investigation suggest that low-intensity running training inhibits the DMH-induced initiation of colon ACF development. **Key Words:** EXERCISE, ACF, 1,2-DIMETHYLHYDRAZINE, COLON CANCER, PRIMARY PREVENTION, PHYSICAL ACTIVITY

Cancer of the large intestine is classified into colon and rectal cancers. The incidence of colon cancer is increasing at a faster rate than that of rectal cancer in recent years in advanced countries, including Japan. Colon cancers develop after the multistep accumulation of genetic and epigenetic induction of oncogenes in both humans and experimental animals (9,14,19).

The proposed multisteps of colon carcinogenesis may start when aberrant crypt foci (ACF) appear in the colon (37). ACF were defined as lesions composed of enlarged crypts, slightly elevated above the surrounding mucosa and more densely stained with methylene blue than normal crypts (3). ACF are considered to be putative preneoplastic colon lesions that may be early indicators of colon carcinogenesis (4,10,18).

Epidemiological evidence has suggested that physical activity has a protective effect on colon cancer incidence (11,13,30). However, few experimental studies have been conducted to elucidate the mechanisms of exercise-related effects on colon cancer. For example, a few earlier animal studies found that both voluntary (1,26) and treadmill (36) running training reduced tumor incidence after the administration of 1,2-dimethylhydrazine (DMH) or azoxymethane. Furthermore, no studies have examined the effects of physical exercise training on colon cancer as they might be related to the multistep nature of colon carcinogenesis, although Demarzo et al. (7) recently reported that a single session of exhaustive exercise increased the number of ACF DMH-induced rat colons. Up to now, there is no study demonstrating that exercise training affects the number of ACF, which is a putative initial step of colon carcinogenesis of rats. Therefore, we investigated the effects of running exercise training on the number of DMH-induced ACF, because previous studies suggested that physical training of this type has a protective effect on colon tumor incidence in rats.

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MATERIALS AND METHODS

Materials

All chemicals, including 1,2-dimethylhydrazine (DMH), a carcinogenic chemical of the colon, was purchased from SIGMA (St. Louis, MO).

Exercise Protocols

Animal care. All experimentation was conducted in accordance with policy statement of the American College of Sports Medicine on research with experimental animals and was approved by the animal care and use committee of National Institute of Health and Nutrition. Four-week-old Fischer 344 male rats were purchased from CLEA Japan, Tokyo. The animals were housed in rooms lighted from 7 a.m. to 7 p.m. and were maintained on an *ad libitum* diet of standard chow and water. The room temperature was maintained at 20–22°C.

Experimental design. After 1 wk of acclimatization to the housing environment (5 wk of age), the rats were randomly assigned to one of two groups: the treadmill-running training group (N = 19) or the control group (N = 19). All rats were given a subcutaneous injection of DMH at a dose level of 20 mg·kg^{-1} body weight, once a week for 2 wk. The DMH was dissolved in 0.1 mM EDTA (pH 6.5) immediately before the administration.

One week after the last injection of DMH (i.e., at age 7 wk), running training was started. The training rats ran for 120 min·d^{-1} (two 60-min bouts separated by 10 min of rest) on a flat motorized treadmill (Natsume, Tokyo, Japan). On the first day, the rats were accustomed to running at a speed of 10 m·min^{-1} by gradually increasing the treadmill speed to the fixed speed. The running speed was maintained for the following 4 wk of training. The intensity of this training was considered to be low because this exercise could be continued for more than 6 h without exhaustion, as reported elsewhere (34).

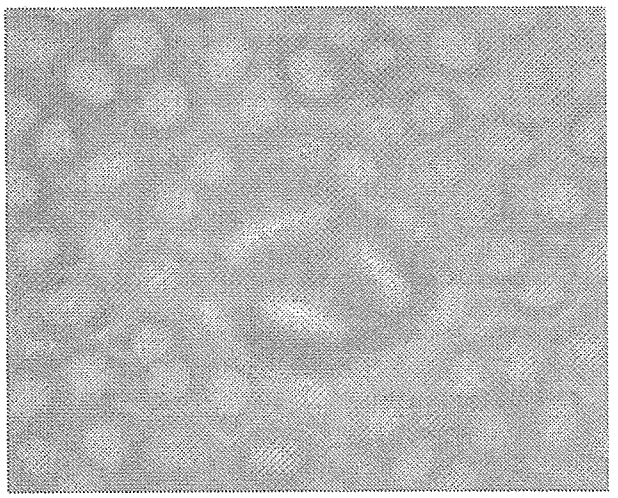

FIGURE 1—1,2-dimethylhydrazine–induced ACF in methylene blue–stained colonic mucosa. In particular, ACF is indicated by the three aberrant crypts per focus that have large crypts, altered luminal openings, and thickened epithelia. This micrograph shows an ACF that consists of three AC. ACF, aberrant crypt foci; AC, aberrant crypts.

TABLE 1. The effect of treadmill-running training on body weight, muscle weight of the heart and soleus, adipose tissue weight of the peritoneum and epididymides, and citrate synthase activity of soleus muscle in rats.

	Control Group (N)	Training Group (N)
BW (g)	216.7 ± 3.5 (19)	199.1 ± 4.0 (19)$^{\#\#}$
Heart weight (mg·g^{-1} BW)	2.89 ± 0.04 (11)	3.00 ± 0.04 (10)$^{\#}$
Soleus weight (mg·g^{-1} BW)	0.33 ± 0.01 (19)	0.37 ± 0.01 (19)$^{\#\#}$
Peritoneal adipose tissue weight (mg·g^{-1} BW)	15.0 ± 0.7 (19)	10.2 ± 0.6 (19)$^{\#\#\#}$
Epididymides adipose tissue weight (mg·g^{-1} BW)	15.1 ± 0.7 (19)	10.2 ± 0.6 (19)$^{\#}$
Brown adipose tissue weight (mg·g^{-1} BW)	1.50 ± 0.07 (11)	1.28 ± 0.10 (10)
Citrate synthase activity in soleus muscle (μmol·g^{-1} wet tissue·min^{-1})	35.9 ± 1.0 (19)	40.3 ± 1.2 (19)$^{\#\#}$

Values are means ± SE. BW, body weight. $^{\#}$,$^{\#\#}$,$^{\#\#\#}$ indicate significant differences from the control group at levels of $P < 0.05$, 0.01, and 0.001, respectively, by t-test.

Two or three days after the last bout of exercise, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg·kg^{-1} body weight), and the heart and soleus muscles were excised, weighed, quickly clamp-frozen in liquid nitrogen, and stored at −80°C until analysis. Then, the colons were dissected and gently flushed with 10% neutralized formalin to remove residual bowel contents, cut open longitudinally, fixed flat between filter papers, and submerged in 10% neutralized formalin overnight at 4°C (23). Peritoneal fat, epididymides fat, and brown adipose tissue (BAT) were excised and weighed.

Detection of ACF. Fixed colons were stained with 0.2% methylene blue, as described by Bird (3). The number of ACF and total number of aberrant crypts (AC) comprising ACF were counted for each colon. The ratio of total AC/ACF was calculated to assess ACF multiplicity. As shown in Figure 1, ACF were identified as lesions composed of enlarged crypts, with an increased pericryptal area, a slightly elevated appearance above the surrounding mucosa with an oval or slitlike orifice, and a higher staining intensity with 0.2% methylene blue than normal crypts (3).

Citrate synthase activity in skeletal muscle. Ten percent homogenates were made from the muscle in 175 mM KCl, 10 mM glutathione, and 2 mM EDTA, pH 7.4. The homogenate was frozen and thawed four times and mixed thoroughly before enzymatic measurements. As a maker of oxidative enzyme, citrate synthase (CS) activity in the soleus muscle was measured using Srere's method (31).

Statistical Analysis

All data are shown as the mean ± SE. Quantitative clinical data were compared between run-trained rats and controls by use of the unpaired t-test. ACF-related data were also compared by use of the Mann–Whitney rank sum test because the numbers of ACF were not normally distributed. These data were analyzed by use of SigmaStat for Windows (SPSS Inc., Chicago, IL). Differences were considered significant when the P value was less than 0.05.

RESULTS

Physical characteristics and citrate synthase activity. The body weight of the training rats was

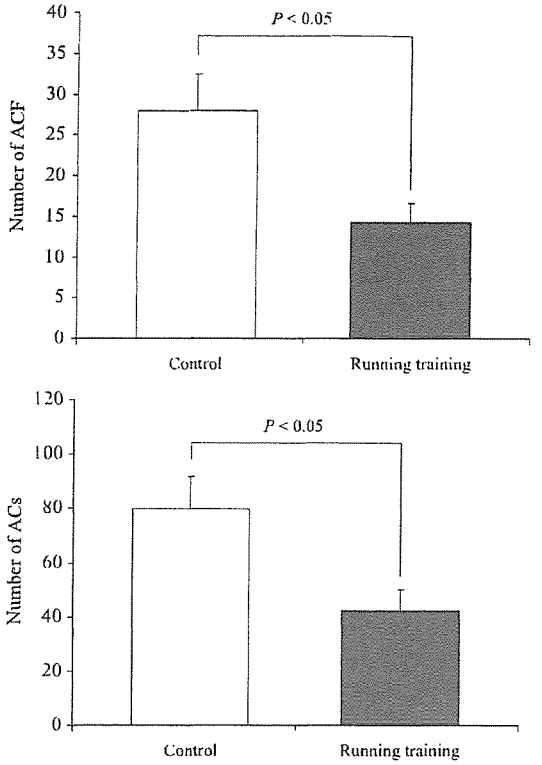

FIGURE 2—Effect of running training on the number of 1,2-dimethylhydrazine–induced ACF (*upper*) and AC (*lower*) in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

significantly less than that of the control rats ($P < 0.01$) (Table 1). The weight of heart and soleus, expressed relative to body weight, was significantly heavier in the training group compared with the control group ($P < 0.05$ and $P < 0.001$, respectively), whereas the relative adipose tissue weight of the peritoneum and epididymides was significantly lighter in the training group compared with the control group ($P < 0.001$ and $P < 0.05$, respectively).

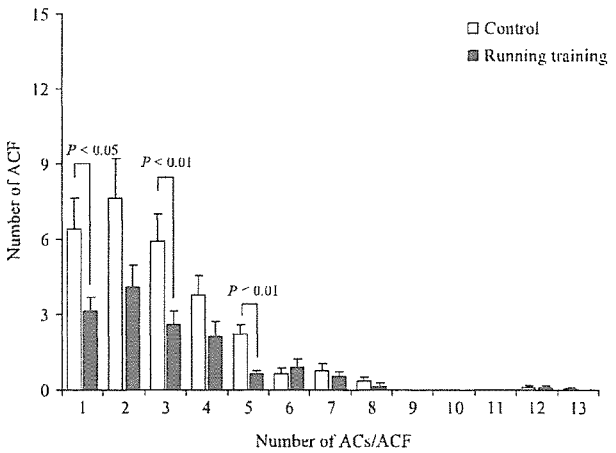

FIGURE 3—Effect of running training on the number of 1,2-dimethylhydrazine–induced AC per focus in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

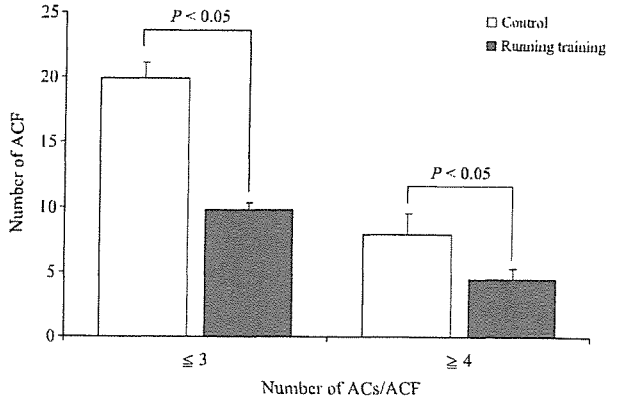

FIGURE 4—Effect of running training on the number of 1,2-dimethylhydrazine–induced small ACF (aberrant crypts per focus ≤ 3) and large ACF (aberrant crypts per focus ≥ 4) in the rat colon. ACF, aberrant crypt foci; AC, aberrant crypts.

The relative BAT weight of the training rats tended to be lower than that of the control group ($P = 0.07$). CS activity in the soleus muscle of the training group was significantly higher than in the control group ($P < 0.05$).

Induction of ACF and AC. As shown in Figure 2 upper panel, the number of ACF of training-group rats was significantly less than that observed in the control group ($P < 0.05$). The number of total AC was also significantly less in the training group than in the control group ($P < 0.05$; Fig. 2 lower panel). As shown in Figure 3, the occurrences of one, three, and five aberrant crypts per focus were significantly smaller in the training group than in the control group ($P < 0.05$). Furthermore, running training decreased the number of not only small ACF, which consists of less than or equal to three AC ($P < 0.05$), but also large ACF (≥ 4 AC) ($P < 0.05$), as compared with the control group (Fig. 4). However, the ratio of total AC/ACF, which indicates the average size of induced ACF, did not significantly differ between the training and control groups (2.9 ± 0.2 vs 2.9 ± 0.7, $P > 0.10$).

DISCUSSION

The main finding of the present study was that short-term, low-intensity running training reduced DMH-induced ACF production in the rat colon.

Colon carcinogenesis is well known to be a multistep process involving multiple genetic alterations (15,37,38). In humans, mutation of adenomatous polyposis coli (APC) gene is regarded as the initial event in ACF, followed by additional mutation of *K-ras* gene in adenomas; further mutation of the *p53* gene is the progressive event in carcinomas (14,33). In rodents, β-catenin mutations are frequently observed in colon tumors and in dysplastic ACF induced by azoxymethane (32). APC and/or K-ras mutations are also occasionally observed in rodents, as they are in humans (32), and ACF has been considered a very initial lesion in a

multistep colorectal tumorigenesis model (14). After ACF were first described in animals, similar lesions were found in humans (28). Because previous studies have suggested that low-intensity, long-term treadmill-running training prevented the incidence or development of colon tumors in a rat model injected subcutaneously with azoxymethane (36), it is reasonable to speculate that exercise training may exert its effect on one or more steps in colon carcinogenesis. To date, however, it remains unknown at which step of the carcinogenic process (e.g., ACF, adenomas (early, intermediate, and late), or carcinomas) physical exercise training exerts its preventive effect in rodents injected with carcinogens. The present results suggest that training in rats suppressed the first step, the initiation of ACF development in the rat colon. This is the first observation regarding the effect of physical exercise on ACF, the development of which is considered the first step in colon carcinogenesis. Furthermore, Colbert et al. (5), using the APC^{Min} mouse model, reported a trend toward fewer polyps in the colon in treadmill-exercised males compared with nonexercised mice. From the present investigation, it is obvious that the earlier phase of colon carcinogenesis is inhibited by exercise training. Therefore, it is of great importance when considering efficient chemopreventive effects on cancer development. Several hypotheses have been suggested to explain the preventive effects of exercise/physical activity on colon carcinogenesis—for example, shortened gastrointestinal transit time as a result of exercise (6); energy balance (16); reduced levels of blood insulin, which might be a growth factor for colon cancer cells (12); enhanced immune activity–related NK cells (20); enhanced free-radical scavenger system (8); changed prostaglandin levels (17); and decreased obesity (25). However, mechanisms related to the exercise-induced decrease in AC and/or ACF are not known at all. Therefore, only a few hypotheses can be raised. First, as Lasko and Bird et al. (16) have reported that caloric restriction–induced weight loss suppressed the increase in the number of ACF after the injection of azoxymethane in rats, it may be possible that energy balance (29), including energy expenditure and reduced food intake (24) or reduced nonexercise activity level (35) by exercise training, may exert a suppressive effect similar to that of caloric restriction, inhibiting the initiation or proliferation of ACF on the colonic mucosa. In fact, the results of the present study indicate that the body weight and/or adipose tissue weight of the peritoneum and epididymides were significantly lower in the running group than in the control group. Therefore, it is plausible that body- or fat weight loss yielded by physical exercise may reduce the initiation of ACF. Another plausible mechanism is that exercise might exhibit its preventive effects on mutation induction in the APC, *K-ras*, and/or *p53* genes through the induction of some detoxification enzymes for oxidative stresses. A third possibility is the commitment of moderate levels of physical exercise on the improvement of lipid metabolism. High fat levels in serum and low expression levels of lipid metabolism–related genes such as lipoprotein lipase in the liver and colon are now considered to have some significant impact on the development of intestinal tumors in the APC^{Min} mouse model (21,22). Further studies are expected to investigate the molecular mechanisms underlying exercise-induced effects on AC/ACF formation.

Recently, Demarzo and Garcia (7) reported that a single bout of exhaustive swimming exercise with a 2% weight tied to the tail was related to an elevated number of colonic ACF in untrained rats treated with DMH, when compared with a control group. Because this report included no description of the exercise protocol, such as a period of acclimatization that is usually given before acute bouts of exercise (27), it is not known how much "stress" was imposed on the exercised rats in the study. Therefore, we could not discuss the different effects of ACF production between the two studies in terms of exercise training–induced stress. On the other hand, the intensity of the swimming exercise with a 2% weight tied to the tail might be higher than that of the running training adopted in the present investigation. Thus, the exercise intensity may be a key factor determining the number of ACF after exercise. In fact, unaccustomed exhaustive and/or high-intensity exercise increases systemic free-radical generation in experimental animals (2). On the other hand, in the present study, we showed that low-intensity physical exercise for 2 h may decrease the development of colonic ACF in experimental animals. Furthermore, stress related to exercise at times during which the rats normally sleep may influence the ACF number in the colon. Therefore, voluntary exercise during the night cycle may be a better alternative exercise protocol than the "forced daytime" treadmill running adopted in the present investigation. However, because the number of ACF in the trained rats in the present study was actually lower than in the nonexercise control group, the overall effects of treadmill running on ACF production are favorable. Therefore, the benefits of the exercise training adopted in the present study are thought to outweigh the disadvantages of exercise-related stress. A future study using voluntary exercise should be conducted to clarify this issue.

The number of AC with a specific number of ACF (1,3,5) in the trained rats was lower than in the control group in the present investigation. However, running training did not affect the overall mean AC/ACF ratios of the rat colon induced by DMH. Thus, it is suggested that the physical exercise training adopted in the present investigation may not be effective for preventing the proliferation of ACF in rat colonic mucosa.

In conclusion, the results of the present investigation demonstrated that low-intensity running training inhibits the initiation of ACF in the rat colon induced by DMH. Furthermore, the present investigation suggests that increasing physical activity might be effective for primary prevention of colon cancer incidence, not only for rats but also for humans, by affecting the first step of cancer induction. However, the clinical implications and pathophysiological mechanisms of these findings warrant further investigation.

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Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates

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Dietary and xenobiotic compounds can disrupt endocrine signaling, particularly of steroid receptors and sexual differentiation. Evidence is also mounting that implicates environmental agents in the growing epidemic of obesity. Despite a long-standing interest in such compounds, their identity has remained elusive. Here we show that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo*. TBT is a dual, nanomolar affinity ligand for both the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor γ (PPARγ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues after organotin, RXR, or PPARγ ligand exposure. TBT represents, to our knowledge, the first example of an environmental endocrine disrupter that promotes adipogenesis through RXR and PPARγ activation. Developmental or chronic lifetime exposure to organotins may therefore act as a chemical stressor for obesity and related disorders. (*Molecular Endocrinology* 20: 2141–2155, 2006)

ORGANOTINS ARE A diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the 1960s as antifouling agents in marine shipping paints, although such use has been restricted in recent years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high-value food crops (1, 2). Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems, and textiles. Mono- and diorganotins are prevalently used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPTO results in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks (3, 4). Bioaccumulation of organotins decreases aromatase activity leading to a rise in testosterone levels that promotes development of male characteristics (5). Imposex results in impaired reproductive fitness or sterility in the affected animals and is one of the clearest examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species (6, 7), but TBT is only reported to have modest adverse effects on mammalian male and female reproductive tracts and does not alter sex ratios (8, 9). Instead, hepatic-, neuro-, and immunotoxicity appear to be the predominant effects of organotin exposure (10). Hence, the current mechanistic understanding of the endocrine-disrupting potential of organotins is based on their direct actions on the levels or activity of key steroid-regulatory enzymes such as aromatase and more general toxicity mediated via damage to mitochondrial functions and subsequent cellular stress responses (11–15).

However, it remains an open question whether *in vivo* organotins act primarily as protein and enzyme

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Abbreviations: Acac, Acetyl-coenzyme A carboxylase; b.w., body weight; C/EBP, CCAAT/enhancer binding protein; 9-*cis* RA, 9-cis retinoic acid; DMSO, dimethylsulfoxide; F, forward; Fatp, fatty acid transport protein; LBD, ligand-binding domain; LXR, liver X receptor; MDIT, 3-isobutyl-1-methylxanthine, dexamethasone, insulin and T_3 adipocyte differentiation mix; PPAR, peroxisome proliferator-activated receptor; R, reverse; RAR, retinoic acid receptor; RXR, retinoid X receptor; Srebf1, sterol-regulatory element binding factor 1; TBT, tributyltin chloride; TPTO, triphenyltin oxide; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid; VDR, vitamin D receptor.

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inhibitors, or rather mediate their endocrine-disrupting effects at the transcriptional level. Recent work has shown that aromatase mRNA levels can be down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, retinoid X receptor (RXRs) or peroxisome proliferator-activated receptor γ (PPARγ) (16–18). Furthermore, Nishikawa *et al.* (19) have demonstrated that the gastropod *Thais clavigara* RXR homolog is responsive to 9-cis-retinoic acid (9-*cis*-RA) and TBT, and 9-*cis* RA can also induce imposex, suggesting a conserved transcriptional mechanism for TBT action across phyla. These ligand-dependent transcription factors belong to the nuclear hormone receptor superfamily—a group of approximately 150 members (48 human genes) that includes the estrogen receptor, androgen receptor, glucocorticoid receptor, thyroid hormone receptor, vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), PPARs, and numerous orphan receptors. We were therefore intrigued by the similar effects of TBT and RXR/PPARγ ligands on mammalian aromatase mRNA expression and hypothesized that TBT might be exerting some of its biological effects via transcriptional regulation of gene expression through activation of one or more nuclear hormone receptors.

Our results show that organotins such as TBT are indeed potent and efficacious agonistic ligands of the vertebrate nuclear receptors, retinoid X receptors (RXRs) and PPARγ. The physiological consequences of receptor activation predict that permissive RXR heterodimer target genes and downstream signaling cascades are sensitive to organotin misregulation. Consistent with this prediction we observe that organotins phenocopy the effects of RXR and PPARγ ligands using *in vitro* and *in vivo* models of adipogenesis. Therefore, TBT and related organotin compounds are the first of a potentially new class of environmental endocrine disrupters that targets adipogenesis by modulating the activity of key regulatory transcription factors in the adipogenic pathway, RXRα and PPARγ. The existence of such xenobiotic compounds was previously hypothesized (20, 21). Our results suggest that developmental exposure to TBT and its congeners that activate RXR/PPARγ might be expected to increase the incidence of obesity in exposed individuals and that chronic lifetime exposure could act as a potential chemical stressor for obesity and obesity-related disorders.

RESULTS

Organotins Are Agonists of Vertebrate RXR and RXR-Permissive Heterodimers

Many known or suspected environmental endocrine-disrupting chemicals mimic natural lipophilic hormones that act through members of the superfamily of nuclear receptor transcription factors (22, 23). In a screen of high-priority endocrine-disrupting chemicals against a bank of vertebrate nuclear receptor ligand-binding domains (LBDs), we observed that organotins, specifically tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), could fully activate an RXRα LBD construct (GAL4-RXRα) in transient transfection assays. Both TBT and TPTO were as potent (EC_{50} ~3–10 nM) as 9-*cis* retinoic acid, an endogenous RXR ligand and approximately 2- to 5-fold less potent than the synthetic RXR-specific ligands LG100268 (EC_{50} ~ 2 nM) or AGN195203 (EC_{50} ~ 0.5 nM) (Fig. 1A and see Table 2). Maximal activation for TBT reached the same levels as LG100268 or AGN195203.

We next tested whether activation by TBT was unique to RXRα only, restricted to RXR heterodimer complexes, or a general nuclear receptor transcriptional response (Fig. 1, B–D, and Table 1). TBT activated RXRα and RXRγ from the amphibian *Xenopus laevis* in addition to human RXRs (Table 1). Our results are consistent with recent findings by Nishikawa *et al.* (19, 24) that organotins promote activation of all three human RXR subtypes in a yeast two-hybrid screen. We also observed significant activation of receptors typically considered to be permissive heterodimeric partners of RXR including human PPARγ (Fig. 1B, ~30% maximal activation of 10 μM troglitazone, but note that activation is compromised by cellular toxicity above 100 nM), PPARδ, liver X receptor (LXR), and the orphan receptor NURR1. In contrast, typical nonpermissive partners such as RARs, thyroid hormone receptor, and VDR failed to show activation by organotins (Fig. 1C and Table 1). Murine PPARα was also not activated by TBT although it was fully activated by its specific synthetic agonist WY-14643 (Fig. 1D). The steroid and xenobiotic receptor was likewise unresponsive. The orphan receptor NURR1, which has no discernable ligand pocket and is believed to be ligand independent (25), was nevertheless activated 7- to 10-fold at 100 nM TBT. Similarly, other RXR-specific ligands, *e.g.* LG100268, activated NURR1 to the same degree, suggesting that this response occurred through NURR1's heterodimeric partner RXR as has been previously described (25, 26). Like other RXR-specific ligands, tributyltin was also able to promote the ligand-dependent recruitment of nuclear receptor cofactors such as receptor-associated coactivator 3 (ACTR), steroid receptor coactivator-1, and PPAR-binding protein in mammalian two-hybrid interaction assays (data not shown). We infer from these results that nuclear receptor activation by TBT activation is specific to a small subset of receptors and not a consequence of a general effect on the cellular transcriptional machinery.

We next investigated the relationship between the structure of the tin compounds and RXR activation by testing the response of GAL4-RXRα to mono-, di-, tri-, and tetra-substituted butyltin, branched side chains, variations in the alkyl chain length, and changes in the halide component (Fig. 1A and Table 2). Overall, trialkyltin compounds were the most effective with nano-

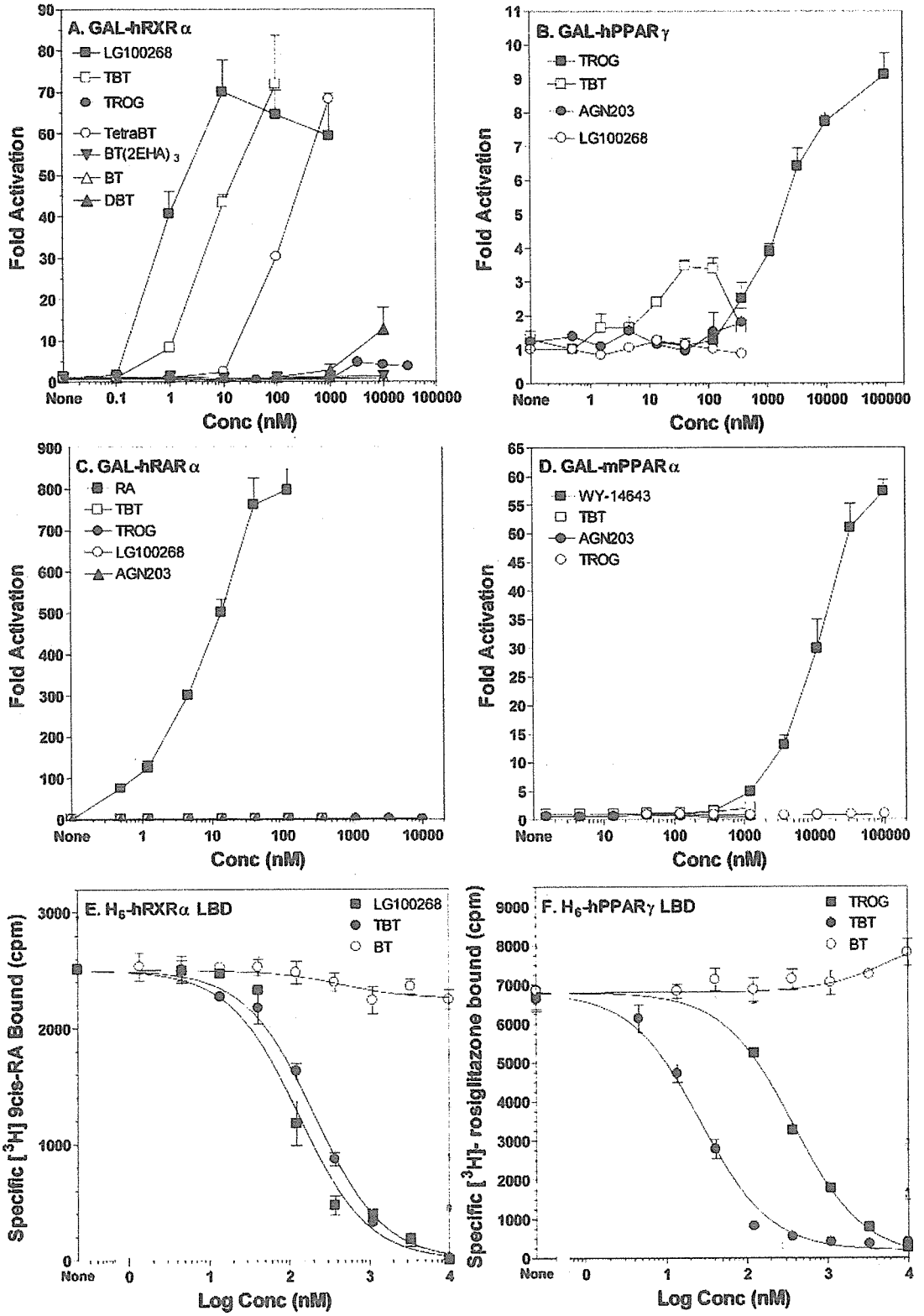

Fig. 1. Organotins Are Agonist Ligands of RXRα and PPARγ

Organotins are high-affinity ligand agonists of RXRα and PPARγ. A–D, Activation of GAL4-hRXRα, -hPPARγ, -hRARα, or -hPPARα in transiently transfected Cos7 cells by organotins and receptor-specific ligands. Data represent reporter luciferase activity normalized to β-galactosidase and plotted as the average fold activation ± SEM (n = 3) relative to solvent-only controls from representative experiments. E and F, Competition binding curves of histidine-tagged RXRα or PPARγ LBDs with TBT. Data shown are from a representative experiment analyzed in GraphPad Prism 4.0 and K_i values deduced (Table 3). Conc, Concentration; DBT, dibutyltin chloride; TROG, troglitazone.

Table 1. TBT Activates RXRs and RXR-Permissive Heterodimers

GAL4-NR LBD	Fold Activation at 60 nM TBT	Permissive RXR Heterodimer
RXRα (*Homo sapiens*)	60	Yes
RXRα (*X. laevis*)	25	Yes
RXRγ (*X. laevis*)	7.0	Yes
NURR1 (*H. sapiens*)	7.0	Yes
LXR (*H. sapiens*)	2.1	Yes
PPARα (*Mus musculus*)	0.7	Yes
PPARγ (*H. sapiens*)	5.3	Yes
PPARδ (*H. sapiens*)	1.7	Yes
RARα (*H. sapiens*)	0.7	No
TR&β (*H. sapiens*)	0.4	No
VDR (*H. sapiens*)	0.5	No
SXR (*H. sapiens*)	1.0	No

Data are fold activation at 60 nM TBT relative to solvent-only controls of transiently transfected Cos7 cells after 24 h ligand treatment. SXR, Steroid and xenobiotic receptor; TR, thyroid hormone receptor.

molar EC_{50} values. Monobutyltin gave no significant activation whereas dibutyltin was moderately active in the micromolar range (Fig. 1A and Table 2). Tetrabutyltin was 20-fold less potent than TBT, whereas the branched side-chain butyltin tris(2-ethylhexanoate) [BT(2-EHA)$_3$] was inactive (Table 2). Although activation by dialkyltins is weaker than that of TBT, it is potentially significant due to their widespread use in the manufacture of polyvinyl chloride plastics and greater solubility than TBT.

The effect of the hydrocarbon chain length was very pronounced, suggesting an important structure-activity relationship. A reduction in hydrophobicity from butyl to ethyl side chains raised the EC_{50} value by almost 1000-fold into the micromolar range. Trimethyltin was weakly active only above 100 μM (Table 2). Substitution of the halide component had no significant effect on the EC_{50} values for TBT, probably due to the lability of the halide atom through exchange in aqueous tissue culture media where chloride ions are prevalent.

Table 2. Organotin EC_{50} Values for Nuclear Receptor LBDs

Ligand	GAL4-NR LBD Transactivation (EC_{50} Values, nM)		
	hRXRα	hRARα	hPPARγ
LGD268	2–5	na	na
AGN195203	0.5–2	na	na
9-*cis* RA	15	na	na
all-*trans* RA	na	8	na
Butyltin chloride	na	na	na
Dibutyltin chloride	3000	na	na
TBT	3–8	na	20
Tetrabutyltin	150	ND	ND
Di(triphenyltin)oxide	2–10	na	20
Butyltin tris(2-ethylhexanoate)	na	ND	ND
Troglitazone	na	na	1000
Tributyltin fluoride	3	ND	ND
Tributyltin bromide	4	ND	ND
Tributyltin iodide	4	ND	ND
Triethyltin bromide	2800	ND	ND
Trimethyltin chloride	>10000	ND	ND

na, Not active; ND, not determined. EC_{50} values were determined from dose-response curves of GAL4-NR LBD construct activation in transiently transfected Cos7 cells after 24-h ligand exposure.

TBT Is a Potent Ligand of Both RXRα and PPARγ

Many, if not most, natural and synthetic nuclear receptor agonists act as ligands that specifically interact with their cognate receptor LBDs. We therefore performed equilibrium competition binding experiments with purified histidine-tagged human RXRα (H_6-RXRα) and PPARγ (H_6-PPARγ) LBDs to determine whether the potent and specific activation of these receptors by TBT was due to direct ligand-receptor interaction (Fig. 1, E and F).

The equilibrium binding curves indicate that TBT is a high-affinity, competitive ligand for 9-*cis* RA-bound RXRα. The inhibition equilibrium dissociation constant was calculated by the Chang-Prusoff method [inhibition constant (K_i) = dissociation constant (K_d)] as 12.5 nM (10–15 nM; 95% confidence interval) (Table 3). By comparison, the value obtained for the synthetic RXR agonist LG100268 was 7.5 nM, which compared favorably with its published value of approximately 3 ± 1 nM (27). Therefore, the identification of TBT as an RXR ligand expands the molecular definition of known rexinoids (agonists able to activate RXR) to include this structurally unique class of organotin compounds.

Somewhat surprisingly, we also observed potent specific competitive binding by TBT for rosiglitazone bound to human PPARγ LBD (Fig. 2B). The deduced K_i of 20 nM (17–40 nM; 95% confidence interval) was slightly higher than for RXRα but significantly better than the K_i for the PPARγ agonist troglitazone, which

Table 3. TBT Binding Constants (K_d) for hRXRα and hPPARγ LBDs

Ligand	Receptor Competitive Inhibition Binding Constants K_i (nM ± 95% CI)		
	H_6-RXRα	H_6-PPARγ	Published
TBT	12.5 (10–15)	20 (17–40)	
LG100268	7.5 (6–10)	ND	3 ± 1[a]
Troglitazone	ND	300 (270–335)	300 ± 30[b]

Competition binding curves were determined at constant ^{3}H-specific ligand concentrations [20 nM 9-*cis*-RA, K_d = 1.4 nM (87) or rosiglitazone, K_d = 41 nM (88)] with increasing cold competitor ligands over the range indicated in Fig. 1, E and F. Data were analyzed in GraphPad Prism by nonlinear regression of a competitive one-site binding equation (Chang-Prusoff method) to determine K_i values ± 95% confidence intervals (n = 3). CI, Confidence interval; ND, not determined.
[a] RXRα:LG100268 K_d = 3 ± 1 nM (27).
[b] PPARγ:troglitazone K_d = 300 ± 30 nM (28).

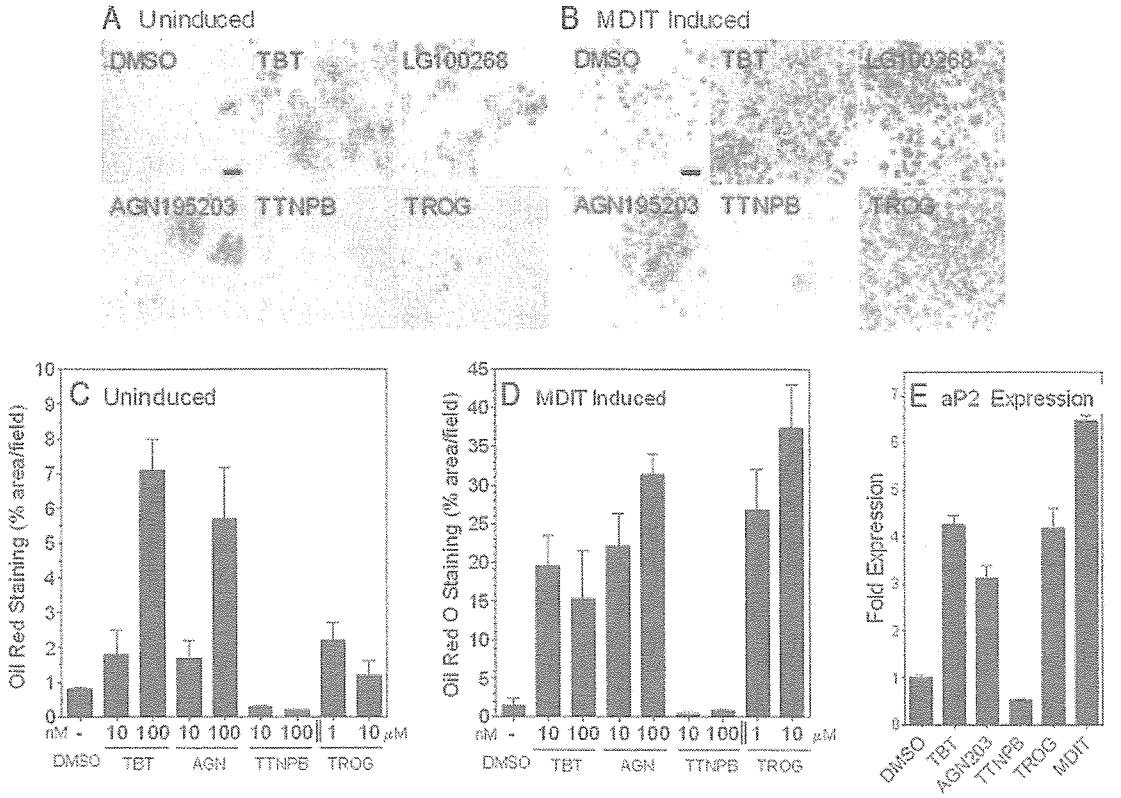

Fig. 2. Tributyltin Induces Adipogenesis in 3T3-L1 Cells

Uninduced (A) and MDIT-induced (B) 3T3-L1 cultures grown for 1 wk in the presence of vehicle (DMSO), or ligands were analyzed for mature adipocyte differentiation by Oil Red O staining. *Scale bar* represents 100 μm. C and D, The percentage area stained was determined by automated analysis of random fields (n = 9) from high-contrast dissecting scope photographs of monolayers analyzed in ImageJ; 1–100 nM of TBT, AGN195203, and TTNPB or 1–10 μM troglitazone. E, Quantitative real-time PCR (QRT-PCR) of adipocyte-specific fatty acid binding protein aP2 (aP2/Fabp4) expression levels in postconfluent 3T3-L1 cells treated with the indicated ligands for 24 h. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase controls and plotted as average fold induction ± SEM (n = 3).TROG, Troglitazone.

yielded a K_i of 300 nM, consistent with its published K_d (28). The K_d values for TBT binding to RXRα (12.5 nM) and PPARγ (20 nM) are also in close agreement with EC_{50} values obtained from transient transfection assays using GAL4-RXRα and GAL4-PPARγ constructs (Table 2).

Taken together, these data show that organotins such as TBT, although structurally distinct from previously described natural or synthetic ligands, can interact with RXRα and PPARγ, via direct ligand binding to induce productive receptor-coactivator interactions and promote transcription in a concentration-dependent manner. Organotins are therefore potent nanomolar receptor activators on par with synthetic RXR and PPARγ ligands such as LG100268, AGN195203, and thiazolidinediones.

TBT Promotes Adipogenesis in the Murine 3T3-L1 Cell (Embryonic Murine Preadipocyte Fibroblast Cell Line) Model

Numerous studies have demonstrated the critical role played by RXRα:PPARγ signaling in regulation of mammalian adipogenesis (29–31). In the murine 3T3-L1 preadipocyte cell model, adipogenic signals induce early key transcriptional regulators such as CCAAT/enhancer binding proteins (C/EBPs) β and δ that lead to mitotic clonal expansion of growth-arrested preadipocytes and induction of the late differentiation factors C/EBPα and PPARγ (32–34). The combination of C/EBPα expression together with PPARγ signaling efficiently drives terminal adipocyte differentiation and lipid accumulation. We therefore tested whether TBT signaling through RXR:PPARγ could promote adipogenesis in the murine 3T3-L1 differentiation assay and compared its effect to other RXR-specific or PPARγ ligands (Fig. 2). Undifferentiated 3T3-L1 cells were cultured for 1 wk in the presence of ligands either with or without a prior 2-d treatment with MDIT (an adipogenic-sensitizing cocktail of 3-isobutyl-1-methylxanthine, dexamethasone, insulin, and T_3) (35). Cells were then scored for lipid accumulation using Oil Red O staining to determine the degree of terminal adipocyte differentiation. TBT was as effective as LG100268 or AGN195203 in promoting dif-

ferentiation in the absence of MDIT treatment, increasing the number of differentiated adipocytes about 7-fold over solvent-only controls (Fig. 2, A and C). The PPARγ agonist troglitazone was a weak inducer in the absence of MDIT. Prior treatment with MDIT increased the response to TBT, LG100268, and AGN195203 a further 3- to 5-fold (Fig. 2, B and D). MDIT treatment also boosted the response to troglitazone to equivalent levels as expected from published studies showing that combination treatment with PPARγ ligands promotes efficient adipocyte differentiation (36–38). In contrast, the RAR agonist TTNPB inhibited the differentiation of 3T3-L1 cells, consistent with previously published data that showed RAR signaling blocks adipogenesis during the early stages of differentiation *in vitro* and can modulate adiposity and whole body weight *in vivo* (39–41). The differential response of 3T3-L1 cells to receptor-selective retinoids indicates that TBT favors RXR homodimer or permissive RXR-heterodimer rather than RXR:RAR signaling in this cell model.

Adipocyte differentiation by TBT was accompanied by direct transcriptional effects on RXR:PPARγ targets such as adipocyte-specific fatty acid-binding protein (aP2) mRNA. The aP2 promoter contains response elements sensitive to C/EBP factors and RXRα:PPARγ signaling (42). Quantitative real-time PCR analysis showed aP2 levels were elevated by TBT treatment approximately 5-fold at 24 h (Fig. 2E) and 45-fold at 72 h (data not shown). LG100268, troglitazone, and MDIT treatment also increased aP2 expression at these time points whereas the RAR agonist TTNPB was inhibitory, consistent with the observed cellular responses.

TBT Induces Adipogenic Regulators and Markers of RXRα:PPARγ Signaling *in Vivo*

The ability of organotins to regulate RXRα:PPARγ target genes and key modulators of adipogenesis and lipid homeostasis *in vivo* has not been previously examined. Therefore, we next asked whether TBT could perturb expression of critical transcriptional mediators of adipogenesis such as RXRα, PPARγ, C/EBPα/β/δ, and sterol regulatory element binding factor 1 (Srebf1) as well as known target genes of RXRα:PPARγ signaling from liver, epididymal adipose tissue, and testis of 6-wk-old male mice dosed for 24 h with TBT [0.3 mg/kg body weight (b.w.)], AGN195203 (0.3 mg/kg b.w.), troglitazone (3 mg/kg b.w), or vehicle (corn oil) administered by ip injection. TBT either had no effect or weakly repressed RXRα and PPARγ transcription in liver (Fig. 3, A and B). A more pronounced decrease was observed for RXRα, PPARγ, C/EBPα, and C/EBPδ in adipose tissue and testis (Fig. 3, B and C). In contrast, TBT, AGN195203, and troglitazone significantly induced expression of the early adipogenic transcription factor C/EBPβ in liver and testis, whereas it was more weakly induced in adipose tissue. Induction was strongest in testis where TBT and troglitazone increased expression greater than 10-fold and AGN195203 increased expression 60-fold compared with vehicle controls (Fig. 3C). In addition to C/EBPβ, the proadipogenic transcription factor Srebf1 was also significantly increased in adipose tissue by all three receptor ligands and weakly induced in liver.

We also observed coordinate changes in several well-characterized direct target genes of RXR:PPARγ signaling. Fatty acid transport protein (Fatp) acts as a key control point for regulation of cellular fatty acid content. The Fatp promoter contains a functional PPAR response element shown to be sensitive to RXR:PPARγ signaling in 3T3-L1 adipocytes and white fat (43–46). Fatp mRNA levels were up regulated 2- to 3-fold in liver and epididymal adipose tissue but not testis by TBT, AGN195203, and troglitazone (see Fig. 5, A and B). Similarly, the PPARγ target phosphoenolpyruvate carboxykinase 1 (PEPCK/Pck1) (47), the rate-limiting step in hepatic gluconeogenesis and adipose glyceroneogenesis, was up-regulated in liver and adipose tissues by TBT or troglitazone treatment.

Signaling through RXR:PPARγ, RXR:LXR, and ADD1/Srebf1 in hepatocytes has been shown to modulate fatty acid synthesis through transcriptional control of acetyl-coenzyme A carboxylase (Acac), the rate-limiting step in long-chain fatty acid synthesis (48, 49), as well as fatty acid synthase (Fasn) (50–53). Hepatic expression of both Acac and Fasn was unregulated between 1.5–2.5-fold by TBT, AGN195203, and troglitazone. Therefore, the coordinate increased expression of Fatp, Pck1, Acac, and Fasn in liver suggests that TBT stimulates fatty acid uptake and triglyceride synthesis. Similar changes have been reported in the induction of hepatic steatosis by overactive PPARγ signaling (49, 54).

Taken together, these data show that TBT exposure induces lipogenic RXR:PPARγ target gene expression, in adipose tissue and liver, and modulates associated early adipocyte differentiation factors such as C/EBPβ and Srebf1. We inferred from these data that organotins are potential adipogenic agents *in vivo*.

Developmental Exposure to TBT Disrupts Lipid Homeostasis and Adipogenesis in Vertebrates

Based on its molecular pharmacology, ability to induce 3T3-L1 adipocyte differentiation, and *in vivo* transcriptional responses, we reasoned that TBT would disrupt normal endocrine control over lipid homeostasis and impact adipogenesis, particularly when exposure occurred during sensitive periods of development. We therefore tested this hypothesis in two vertebrate model systems, mouse and *X. laevis*, during embryogenesis.

Pregnant C57BL/6 mice were injected daily from gestational d 12–18 with TBT (0.05 or 0.5 mg/kg body weight ip) dissolved in sesame oil or vehicle alone. Pups were then killed at birth, and histological sections were prepared from liver, testis, mammary gland, and inguinal adipose tissue. Sections were stained