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Effects of estrogens and metabolites on endometrial carcinogenesis in young adult mice initiated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine

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Abstract

The present study assessed effects of estrogens and their steroid metabolites on the endometrial carcinogenesis in young adult mice initiated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG). A total of 272 female CD-1 (ICR) mice were used and equally divided into 17 groups. Mice were implanted cholesterol pellets to the back subcutis at 9 weeks of age. Pellets contained nothing (control) or one of the experimental agents, three different estrogens and their 13 different steroid metabolites, at a concentration of 0.5% (w/w). At 10 weeks of age, mice were given a single intra-uterine administration of ENNG at a dose of 25 mg/kg body weight. When reaching the 30 weeks of age (20 weeks after the ENNG treatment), mice were sacrificed to assess the development of endometrial proliferative lesions. While endometrial proliferative lesions, including hyperplasias and adenocarcinomas, were observed in all groups, the incidences of hyperplasias in the groups treated with 2-hydroxyestriol, 2-methoxyestradiol, 2-methoxyestriol and 16-epiestriol were significantly higher than that in the control group. On the other hand, adenocarcinomas were significantly developed in the groups treated with estrone, estradiol, estriol, 16β-hydroxyestrone, 16α-hydroxyestrone and 17-epiestriol. These results indicate that, on the endometrial carcinogenesis in mice initiated with ENNG, estrogens and their metabolites belonging to the 16α-hydroxylation pathway and the upstream of the 16β-hydroxylation pathway exert both promoting and progressing effects, whereas, the estrogen metabolites belonging to the 2- and 4-hydroxylation pathways (catechol estrogens) and the downstream of the 16β-hydroxylation pathway exert only promoting or no effects. It is thus suggested that a metabolic profile of estrogens may be crucial for the endometrial carcinogenesis and that the rate of the 16α-hydroxylation may be associated with the increased carcinogenic risks of estrogens on the endometrium.

Keywords: Endometrial carcinogenesis; Estrogen metabolites; *N*-Ethyl-*N*′-nitro-*N*-nitrosoguanidine; Mice

1. Introduction

Estrogens are mainly metabolized in the liver through cytochrome *P*-450-dependent hydroxylation at the C-2, C-4, C-16α or C-16β position to provide two categories of their metabolites, catechol estrogens

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(2- or 4-hydroxylated products) and 16α- or 16β-hydroxylated products, and profiles of such a metabolism play pivotal roles in the biological actions of estrogen [1,2]. While estrogens are involved in the background mechanisms underlying a variety of physiological and pathological phenomena in humans as well as animals, it is widely recognized that estrogens are also critically involved in the development of uterine and mammary malignancies [3–5]. It is thus easily expected that the estrogen metabolism is really important to understand the carcinogenic mechanisms for such organs and, in turn, to draw strategies to control such cancers.

Catechol estrogens have been shown to be weakly antiestrogenic and nongenotoxic, while 16α-hydroxyestrone is potently estrogenic, as well as genotoxic and capable of inducing aberrant proliferation, towards human or mouse mammary tumor cell lines [6,7]. Among mouse strains, the increase of the 16α-hydroxylation rate of estrogens correlates with the increase of the frequency of spontaneous development of mammary tumors and the presence of mammary tumor virus [8]. Conversely, when hepatic 2-hydroxylation, but not 16α-hydroxylation, of estrogens is enhanced by indole-3-carbinol, the spontaneous generation of mammary tumors is inhibited in mice [9]. In humans, an urinary ratio of 16α-hydroxyestrone to 2-hydroxyestrone is suggested to correlate to a risk for the development of breast cancers in pre- and post-menopausal women [10–12]. Furthermore, the estrogen-16α-hydroxylase activity is increased in women with mammary cancers [13]. It is thus indicated that the 16α-hydroxylation pathway of the estrogen metabolism is critically involved in mammary carcinogenesis, and the increased formation of 16α-hydroxylated estrogen products may increase the risk of development of breast cancers in both mice and humans. In contrast, little is known for the biological significances of the 16β-hydroxylation pathway of the estrogen metabolism and its byproducts, especially those regarding carcinogenesis.

Whereas similar findings have been published in the literature also for endometrial carcinogenesis [13,14], information is so limited that the biological meanings of a profile of the estrogen metabolism in the development of endometrial cancers still remain largely obscure. Such limited information is partly due to the limited availability of suitable animal models, and we have made large efforts to develop mouse and rat models to induce endometrial adenocarcinomas with relatively high incidences featuring not too complicate manipulations [15]. In these contexts, the present study was conducted to assess effects of estrogens and their steroid metabolites on the endometrial carcinogenesis in young adult mice initiated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG).

2. Materials and methods

2.1. Animals and chemicals

A total of 272 female CD-1 (ICR) mice, 4 weeks old, were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan), housed four each in a plastic cage with white flake bedding and kept in an air-conditioned room at the constant temperature of 21 ± 2 °C and the controlled relative humidity of 55 ± 10% under a 12 h light/12 h dark cycle. ENNG was purchased from Nacalai Tesque Inc. (City of Kyoto, Kyoto, Japan) and dissolved at a concentration of 1.5% (w/v) in polyethylene glycol before use. Estrogens and their steroid metabolites (all obtained from Sigma Chemical Co., St Louis, MO, USA) used were estrone [1,3,5(10)-estratrien-3-ol-17-one] (E1), estradiol [1,3,5(10)-estratrien-3,17β-diol] (E2), estriol [1,3,5(10)-estratrien-3,16α,17β-triol] (E3), 2-hydroxyestrone [1,3,5(10)-estratrien-2,3-diol-17-one] (2-OH-E1), 2-hydroxyestradiol [1,3,5(10)-estratrien-2,3,17β-triol] (2-OH-E2), 2-hydroxyestriol [1,3,5(10)-estratrien-2,3,16α,17β-tetrol] (2-OH-E3), 2-methoxyestrone [1,3,5(10)-estratrien-2,3-diol-17-one 2-methyl ether] (2-M-E1), 2-methoxyestradiol [1,3,5(10)-estratrien-2,3,17β-triol 2-methyl ether] (2-M-E2), 2-methoxyestriol [1,3,5(10)-estratrien-2,3,16α,17β-tetrol 2-methyl ether] (2-M-E3), 4-hydroxyestrone [1,3,5(10)-estratrien-3,4-diol-17-one] (4-OH-E1), 4-hydroxyestradiol [1,3,5(10)-estratrien-3,4,17β triol] (4-OH-E2), 16β-hydroxyestrone diacetate [1,3,5(10)-estratrien-3,16β-diol-17-one diacetate] (16β-OH-E1), 16-epiestriol [1,3,5(10)-estratrien-3,16β,17β-triol] (16-epi-E3), 16,17-epiestriol [1,3,5(10)-estratrien-3,16β,17α-triol] (16,17-epi-E3), 16α-hydroxyestrone [1,3,5(10)-estratrien-3,16α-diol-17-one] (16α-OH-E1) and 17-epiestriol

[1,3,5(10)-estratrien-3,16α,17α-triol] (17-epi-E3). Their chemical structures are shown in Fig. 1 as a metabolic map. For administration purpose, a pellet was prepared which contains 0.16 mg of one of the above agents and 31.84 mg of cholesterol to make an agent of 0.5% (w/w) concentration by the method described previously [16].

2.2. Animal treatments and assessments

The animal experiment had been approved by the Animal Experimentation Committee of Sasaki Institute prior to the execution and was conducted under the monitoring by the committee in accordance with the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105 and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

The animals were equally divided into 17 groups at 9 weeks of age for experimentation, when they were implanted cholesterol pellets containing nothing (group 1, control), estrogens or their metabolites (groups 2–17, see Fig. 1 and Tables 1 and 2) to the back subcutis. The pellets were renewed every 7 weeks throughout the experiment. At 10 weeks of age, each mouse was given a single dose of ENNG (12.5 mg/kg body weight) into one of the uterine cavities using a 23-gauge needle (45 mm in length) via the vagina. The experiment was terminated at 30 weeks of age (20 weeks after the ENNG treatment). Upon termination, all the surviving animals were autopsied to obtain female reproductive organs with the uterus and ovaries weighed. The excised organs were then fixed in 10% neutrally buffered formalin. Each fixed uterus specimen was cut into 5–7 sections, transversely from the uterine horns to the corpus uterine, and the other fixed organs were appropriately cut longitudinally. These sections were processed through a routine hematoxylin-and-eosin staining procedure for the histological examination.

Mice were allowed free access to a laboratory chow (CRF-1, Oriental Yeast Corp. Ltd, Itabashi, Tokyo, Japan) and tap water throughout the acclimation and experimental periods. Body weights, food intake and water consumption were monitored weekly. Vaginal smears were taken every morning (8:00–9:00) to check the estrus cycle between the 24 and 28 weeks of age, and the number of mice with persistent estrus observed during the period were obtained for all groups to calculate percent incidences.

The endometrial proliferative lesions were histologically classified into two categories, hyperplasia (Fig. 2A) and adenocarcinoma (Fig. 2B), and hyperplasias were further classified based on the degree of cellular atypism into three subcategories of slight (+), moderate (++) and severe (+++), according to the criteria described elsewhere [16,17]. Differential diagnosis between severe hyperplasias and adenocarcinomas is frequently difficult, but the criteria tells that irregular proliferation of atypical glands are observed in the endometrium without invading into the myometrium in severe hyperpasias, whereas irregularly proliferating cells with more prominent atypia form one or more columnar layers with clear evidence of invasion into the myometrium in adenocarcinomas. Such differential diagnosis was made not only by the presence or absence of the invasion but also by the other parameters including the grades of cellular and structural atypia and the frequency of mitoses, but it has been well recognized that the presence or absence of the invasion is the most important parameter to distinguish adenocarcinomas from hyperplasias. In addition to hyperplasias and adenocarcinomas, we diagnosed a lesion showing focal or multifocal downgrowth of glands into the myometrium with stromal components as adenomyosis [16]. Number of mice baring the most malignant lesions among the above categories were obtained for all groups to calculate percent incidences.

2.3. Statistics

Dunnet multiple comparison test was used to assess statistical significance of inter-group differences of means after one-way analysis of variance to determine variations among group means, followed by Bartlett's test to determine homogeneity of variance. Fisher's exact test was used to assess statistical significance of inter-group differences of the incidences. To assess the relationships between two specified factors, Pearson's correlation coefficients (r) and regression functions were obtained by linear regression analyses. The statistical significance was determined, when the p value was less than 0.05.

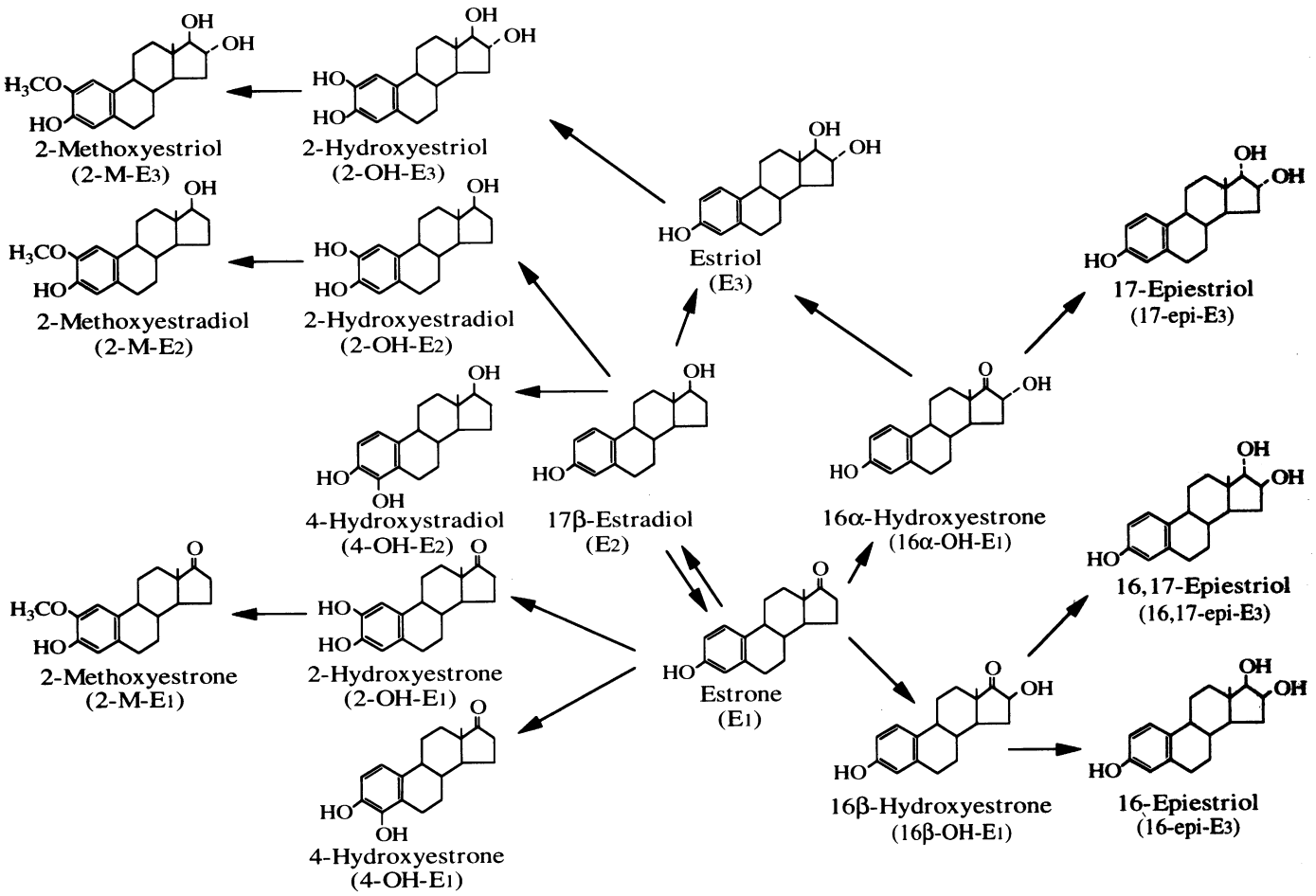

Fig. 1. Chemical structures and the metabolic map of estrogens and their steroid metabolites used in the present study.

Table 1
Final body and relative organ weights, estrogenic potency and incidence of persistent estrus

Group	Treatment	Effective number of animals	Final body weight (g)	Relative organ weight (mg/g body weight)		Estrogenic potency	Incidence of persistent estrus
				Ovaries	Uterus		
1	Control	16	43.9 ± 5.8[a]	0.63 ± 0.13[a]	4.83 ± 1.43[a]	0 [b]	0 (0) [c]
2	E1	16	40.1 ± 6.1	0.37 ± 0.16[d]	10.21 ± 2.05[d]	112	16 (100)[d]
3	E2	16	39.6 ± 6.7	0.36 ± 0.17[d]	11.13 ± 3.11[d]	131	16 (100)[d]
4	E3	16	42.3 ± 4.0	0.35 ± 0.12[d]	8.19 ± 3.31[d]	70	16 (100)[d]
5	2-OH-E1	16	46.3 ± 6.7	0.63 ± 0.19	5.84 ± 3.87	21	2 (13)
6	2-OH-E2	16	45.6 ± 5.4	0.76 ± 0.26	6.01 ± 3.29	24	1 (6)
7	2-OH-E3	16	44.9 ± 5.7	0.68 ± 0.21	5.48 ± 1.56	14	2 (13)
8	2-M-E1	16	44.2 ± 5.3	0.59 ± 0.20	5.34 ± 2.50	11	3 (19)
9	2-M-E2	16	44.2 ± 5.7	0.73 ± 0.20	4.54 ± 1.69	-6	5 (31)
10	2-M-E3	16	41.2 ± 6.1	0.74 ± 0.21	5.07 ± 1.74	5	4 (25) [4]
11	4-OH-E1	15	42.9 ± 5.1	0.65 ± 0.13	5.66 ± 1.72	17	1 (7)
12	4-OH-E2	16	41.6 ± 6.5	0.72 ± 0.19	5.78 ± 2.46	20	7 (44) [d]
13	16β-0H-E1	15	43.1 ± 4.8	0.46 ± 0.11	7.31 ± 2.25	51	15 (100) [d]
14	16-epi-E3	16	43.8 ± 6.6	0.55 ± 0.18	5.98 ± 1.76	24	16 (100) [d]
15	16,17-epi-E3	16	44.4 ± 5.9	0.64 ± 0.18	4.84 ± 1.85	0	2 (13)
16	16α-OH-E1	15	40.4 ± 6.9	0.48 ± 0.18	7.96 ± 2.96[d]	65	12 (80) [d]
17	17-epi-E3	15	39.7 ± 5.6	0.40 ± 0.15[d]	9.30 ± 2.02[d]	93	14 (93)[d]

[a] Means ± SD.
[b] Percent increase of the relative uterine weight over the group 1 value.
[c] Numbers of applicable animals with percent incidences in the parentheses.
[d] Significantly different from the group 1 value.

Table 2
Incidences of endometrial proliferative lesions

Group	Treatment	Effective number of animals	Endometrial proliferative lesions Hyperplasias				Adenocarcinomas	Total
			+	++	+++	Total		
1	Control	16	2 (13)[a]	6 (38)	1 (6)	9 (56)	0 (0)	9 (56)
2	E1	16	1 (6)	1 (6)	2 (13)	4 (25)	12 (75)[b]	16 (100)[b]
3	E2	16	0 (0)	2 (13)	1 (6)	3 (19)	13 (81)[b]	16 (100)[b]
4	E3	16	1 (6)	7 (44)	2 (13)	10 (63)	6 (38)[b]	16 (100)[b]
5	2-OH-E1	16	5 (31)	6 (38)	2 (13)	13 (81)	0 (0)	13 (81)
6	2-OH-E2	16	7 (44)	4 (25)	1 (6)	12 (75)	0 (0)	12 (75)
7	2-OH-E3	16	6 (38)	8 (50)	1 (6)	15 (93)[b]	0 (0)	15 (93)[b]
8	2-M-E1	16	7 (44)	3 (19)	0 (0)	10 (62)	0 (0)	10 (62)
9	2-M-E2	16	7 (44)	7 (44)	1 (6)	15 (93)[b]	0 (0)	15 (93)[b]
10	2-M-E3	16	8 (50)	8 (50)	0 (0)	16 (100)[b]	0 (0)	16 (100)[b]
11	4-OH-E1	15	4 (27)	5 (33)	1 (7)	10 (67)	0 (0)	10 (67)
12	4-OH-E2	16	6 (38)	4 (25)	2 (13)	12 (75)	0 (0)	12 (75)
13	16β-0H-E1	15	2 (13)	7 (47)	2 (13)	11 (73)	4 (27)[b]	15 (100)[b]
14	16-epi-E3	16	9 (56)[b]	5 (31)	2 (13)	16 (100)[b]	0 (0)	16 (100)[b]
15	16,17-epi-E3	16	5 (31)	6 (38)	1 (6)	12 (75)	0 (0)	12 (75)
16	16α-OH-E1	15	1 (7)	6 (40)	4 (27)	11 (73)	4 (27)[b]	15 (100)[b]
17	17-epi-E3	15	1 (7)	2 (13)	1 (7)	4 (27)	11 (73)[b]	15 (100)[b]

[a] Numbers of baring animals with percent incidences in the parentheses.
[b] Significantly different from the group 1 value.

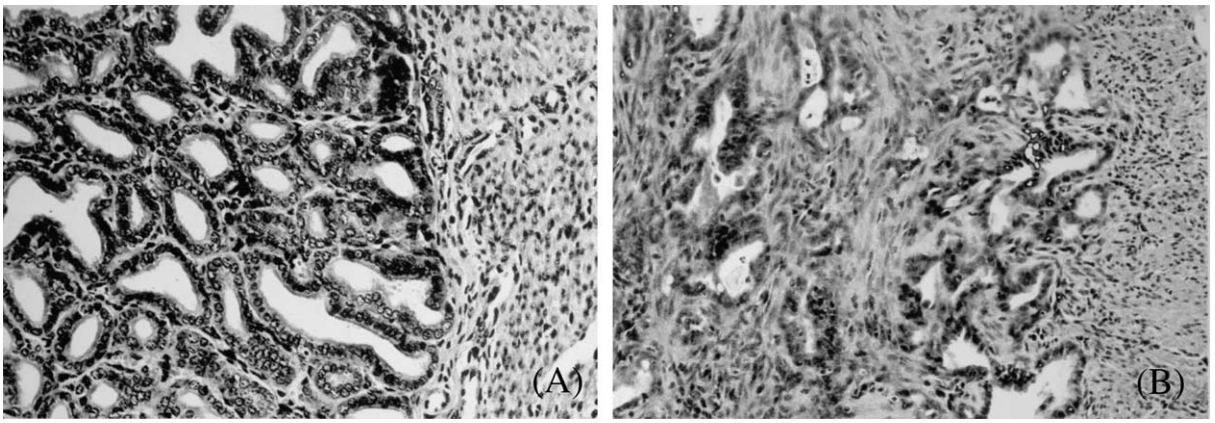

Fig. 2. Representative histology of the endometrial proliferative lesions. (A) Hyperplasia+++ and (B) adenocarcinoma (hematoxylin-and-eosin, X 125).

3. Results

3.1. Final body and relative organ weights, estrogenic potency and incidence of persistent estrus

The body weights, food intake and water consumption did not differ among groups throughout the experimental period (data not shown), and in turn there were no inter-group differences in terms of the final body weight (Table 1). Relative weights of ovaries were significantly lighter in groups 2, 3, 4 and 17 than that of group 1, while relative uterus weights were significantly heavier in groups 2, 3, 4, 16 and 17 than that of group 1 (Table 1). When the estrogenic potency is defined as a percent increase of the relative uterine weight over the group 1 value to reflect the uterotropic effects of the agents, E1, E2, E3, 16β-OH-E1, 16α-OH-E1 and 17-epi-E3 (groups 2, 3, 4, 13, 16 and 17, respectively) were strongly estrogenic with the values over 50%, while the estrogenic potencies of the other estrogen metabolites were weak or absent (Table 1). In contrast, the incidences of the persistent estrus were significantly higher in groups 2, 3, 4, 10, 12, 13, 14, 16 and 17 than that in group 1 (Table 1).

3.2. Histological findings and incidences of endometrial proliferative lesions

There were no macroscopic changes in the organs in particular other than the female reproductive system. Histologically, ovarian atrophy was prominent in mice of groups 2, 3, 4, 13, 16 and 17. In uteri, endometrial proliferative lesions (see Fig. 2 for their representative histology) were induced in some or all animals of every group. Probably because the administration of ENNG was conducted into one of the uterine cavities, severity and incidences of such lesions, when observed, were always higher in one of the cavities than in the other. Incidences of total hyperplasias in groups 7, 9, 10 and 14 were significantly higher than that in group 1, whereas adenocarcinomas were significantly induced in groups 2, 3, 4, 13, 16 and 17 (Table 2). A few adenomyoses were found in groups 1 (1/16, 6.3%), 2 (2/16, 12.5%), 4 (1/16, 6.3%), 6 (1/16, 6.3%), 7 (2/16, 12.5%), 9 (1/16, 6.3%), 13 (1/15, 6.7%), 15 (2/16, 12.5%) and 16 (1/15, 6.7%), respectively, but not in groups 3, 5, 8, 10, 11, 12, 14 or 17. There were no significant differences for the incidences of adenomyoses among experimental groups.

3.3. Correlations between the estrogenic potency or the induction of persistent estrous and the development of endometrial adenocarcinomas

The correlation between the estrogenic potencies and the incidence of endometrial adenocarcinoma were extremely significant among groups ($r = 0.9663$ with the 95% confidence interval (CI) between 0.9068 and 0.9880, $p < 0.0001$) (Fig. 3A). Whereas the incidence of persistent estrus was also significantly correlated with that of endometrial adenocarcinoma

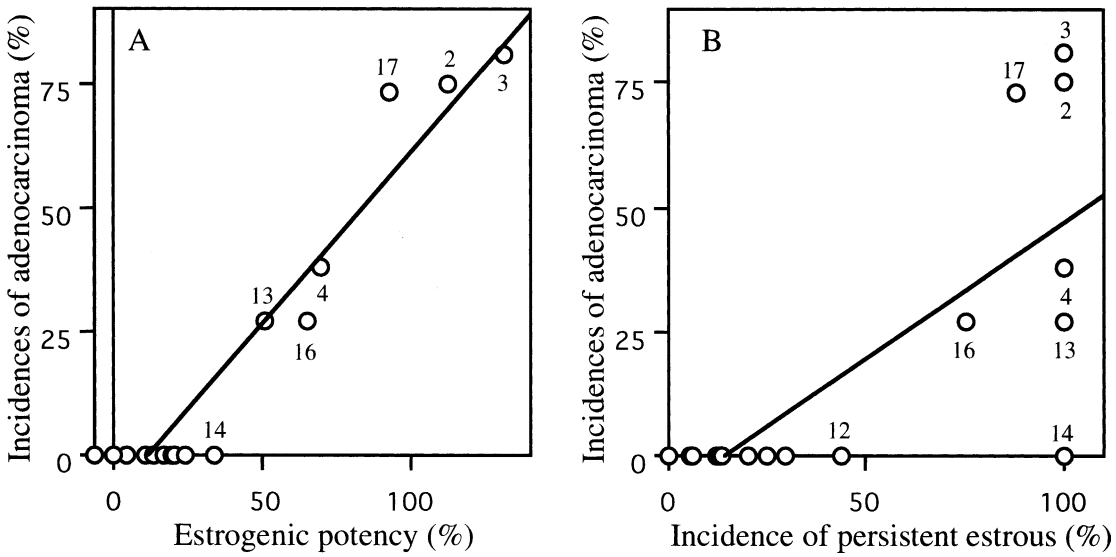

Fig. 3. Correlation between (A) the estrogenic potency or (B) the incidence of persistent estrous and the incidence of endometrial adenocarcinoma. Circle represents each group value, and numbers beside circles are group numbers.

($r = 0.7589$ with the 95% CI between 0.4379 and 0.9083, $p = 0.0004$) (Fig. 3B), this correlation was apparently weaker than the above.

4. Discussion

The present results indicate that estrogens and their steroid metabolites affect endometrial carcinogenesis in mice initiated with ENNG in a manner dependent on their metabolic attributes. Estrogens (E1, E2 and E3) and their metabolites belonging to the 16α-hydroxylation pathway (16α-OH-E1 and 17-epi-E3) and the upstream of the 16β-hydroxylation pathway (16β-OH-E1) exert both promoting and progressing effects on the ENNG-initiated mice endometrial carcinogenesis as evidenced by the development of frank adenocarcinomas. On the other hand, the estrogen metabolites belonging to the downstream of the 16β-hydroxylation pathway are shown to exert only promoting effect on this model of endometrial carcinogenesis as evidenced by the enhanced development of hyperplasias (16-epi-E3), or not to affect (16,17-epi-E3). The inferior potency of the effects of 16α-OH-E1 or 16β-OH-E1 to those of E1 is conceivably due to the addition of a hydroxyl group to the 3β-OH-17-one skeleton of E1 (see Fig. 1 and Table 2). Similarly the different potencies of the effects seen among 3β,16,17-hydroxy-estratrien groups (E3. 16-epi-E3, 16,17-epi-E3 and 17-epi-E3) are conceivably due to the structural differences among these stereoisomers; i.e. the presence of a hydroxyl group at the 16β position (see Fig. 1 and Table 2). For instance, Bradlow et al. [18] reported that E3 and 17-epi-E3 easily form covalent adducts to protein including an estrogen receptor molecule. Regarding the estrogen metabolites belonging to the 2- and 4-hydroxylation pathways (catechol estrogens), in contrast, some of them (2-OH-E3, 2-M-E2 and 2-M-E3) can exert promoting, but not progressing, effects, while the rest of them (2-OH-E1, 2-OH-E2, 2-M-E1, 4-OH-E1 and 4-OH-E2) do not affect the endometrial carcinogenesis in mice initiated with ENNG. These different actions among the catechol estrogens may also be due to the difference of solid configuration among such estrogen metabolites (see Fig. 1 and Table 2). It is thus indicated that the 16α-hydroxylation pathway is most critical in the involvement of estrogens in the endometrial carcinogenesis, which is in line with the limited publications present in the literature [13,14]. This also provides the idea that the way of the involvement of estrogens in

the endometrial carcinogenesis is similar to that in the mammary carcinogenesis (see Section 1), and thus the strategy to control these cancers can be drawn in a unified manner.

The accumulated data obtained from rodent experiments have made it well recognized that a hormonal imbalance leading to the increase in ratio of estrogen to progesterone is an important driving force [19,20]. In fact, the earlier and higher occurrence of persistent estrous in rats as a result of the early onset of chronic hormonal imbalance enhances the development of endometrial adenocarcinomas in rats [15]. The present study revealed, however, that magnitude of the pro-carcinogenic effects of estrogens and their steroid derivatives closely correlate with their uterotropic ('estrogenic') strength, more relevant than their capability to induce persistent estrous. We have shown that the artificially introduced hormonal imbalance is not in itself enough for the induction of endometrial carcinomas in rodents [16,21]. It is, therefore, suggested that, while chronic hormonal imbalance is indeed important, the estrogenic potencies of chemicals may cause more direct impact towards the endometrial carcinogenesis.

Catechol estrogens (2- or 4-OH-E2) induces endometrial adenocarcinomas in mice when neonatally administered [22] but not when post-natally administered [17], in contrast to E2 that can induce such cancers regardless of the administrating age, neonatal or post-natal periods [17,23]. Similar phenomena have been observed also with regard to tamoxifen and diethylstilbestrol [24–27]. It is thus conceivable that catechol estrogens may behave similarly to such compounds regarding the endometrial carcinogenesis in mice, and our previous [28] and present results suggest that these chemicals might act as estrogen agonistic or antagonistic, depending upon the background statuses of endogenous estrogens and their related hormones or of the cellular signal transduction pathways, including that downstream of estrogen receptors, in the endometrium.

In conclusion, a metabolic profile of estrogens may be crucial for the endometrial carcinogenesis, and the rate of the 16α-hydroxylation may be associated with the increased carcinogenic risk of estrogens on the endometrium.

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