TABLE 2.—Summary of histopathological changes up to five weeks of age.

Age/organs/findings	Groups (μg/kg)					
	DES 0	DES0.15	DES1.5	DES15	DES150	DES1500
PND 14 (response to R3-4)						
Number of glands/section	4.3 ± 1.1	4.7 ± 1.5	3.1 ± 1.1	4.0 ± 1.8	4.2 ± 0.8	5.8 ± 0.6
Vagina: cornification	–[a]					+[b]
PND 21						
Ovary: small	–	–	–	–	–	+
5 weeks						
Ovary: atrophy with atretic follicles and no CL	–	–	–	–	+/–[c]	+
Uterus: intraluminal hyperplasia in the surface epithelium	–	–	–	–	–	+
Vagina: cornification	–	–	–	–	–	+

Abbreviations: CL, corpus luteum; DES, diethylstilbestrol; PND, postnatal day.
[a] No abnormalities detected in any animals.
[b] Detected in all animals.
[c] Detected in only some of the animals.

(GAPDH) mRNA transcription were examined using 400-ng aliquots of total RNA. The primers were synthesized and purified by Takara Bio Inc. (Shiga, Japan) based on previous reports (Azabo et al. 2000; Pillai et al., 2002). Expressed levels of ERα, ERβ and PR mRNA relative to GAPDH mRNA expression were compared among the control and the treated groups.

Statistical Analysis

Values for incidences were statistically analyzed using the Fisher exact probability test. Other data were assessed using analysis of variance (three groups) and *t* test (two groups), and post hoc comparisons between the treated and control groups were made with the Dunnett *t* test. Statistical significance was considered as $p < .05$.

RESULTS

Estrogenic Activity in Experiment 1

The results of uterotrophic assays are summarized in Table 1. Both the absolute and relative uterine weights were significantly increased at 1.5 μg/kg and above with dose dependence, except at the highest dose. The uterine weights at the lowest dose were comparable to those in the control group.

Clinical Observation in Life and Estrous Cyclicity in Experiment 2

No treatment-related clinical signs were observed at any time point. Body weight changes were comparable in all groups, including the controls (data not shown). The timing of VO was not different between the treated groups and control. The age at VO was 28.0 ± 1.0, 28.0 ± 0.0, 28.2 ± 0.4, 28.5 ± 0.5, 28.9 ± 1.2, 28.8 ± 1.3 in the 0, 0.15, 1.5, 15, 150, and 1,500 μg/kg groups, respectively.

Data for the estrous cycle are shown in Figure 1. At 1,500 μg/kg, most rats already showed PE at the start of checking estrous cycles, and all were affected by seven weeks of age. At 150 μg/kg, a few females demonstrated PE at five weeks of age, and most by seventeen weeks of age. At 15 or 1.5 μg/kg, PE rates began to increase at thirteen or fifteen weeks of age, and most of them reached PE by nineteen or twenty-one weeks of age, respectively. In all these groups, the numbers of rats exhibiting PE were significantly increased compared to that of controls at fifteen to thirty-one weeks and seventeen to twenty-one weeks of age, respectively. At twenty-three weeks of age, PE began to increase in the control group, and most animals were affected by thirty-nine weeks of age. In the 0.15 μg/kg group, the lowest dose group, the onset of PE was similar to that in controls.

Morphological Changes up to Five Weeks of Age in Experiment 2

Changes in the ovary, uterus, or vagina at PND14, PND21, or five weeks of age are summarized in Table 2, and characteristic lesions are shown in Figures 2, 3, and 4, respectively. Although no abnormalities were detected in the ovary at PND14, those at 1,500 μg/kg were clearly small at PND21 (Figure 2). At five weeks of age, the ovaries of all rats at 1,500 μg/kg and some of rats at 150 μg/kg were macroscopically small in size and microscopically composed of cystic atretic follicles, and they lacked corpora lutea. No hyperplasia or hypertrophy of the interstitial cells was found in the ovaries of all treated groups. In the uterus, there were no differences until PND21, with normal gland genesis at PND14. At five weeks of age, the uteri at 150 μg/kg and higher doses showed intraluminal hyperplasia (Figure 3). The uteri in the other groups showed normal morphology at estrus. In the vagina, cornification was detected in the highest dose group at PND14 (Figure 4). At PND21, this change was recovered to several layered squamous cells similar to those of control animals. At five weeks of age, it was a feature in all rats at 1,500 and most of those in the 150 μg/kg group. The vaginas in the 15 μg/kg and lower groups showed morphology similar to those

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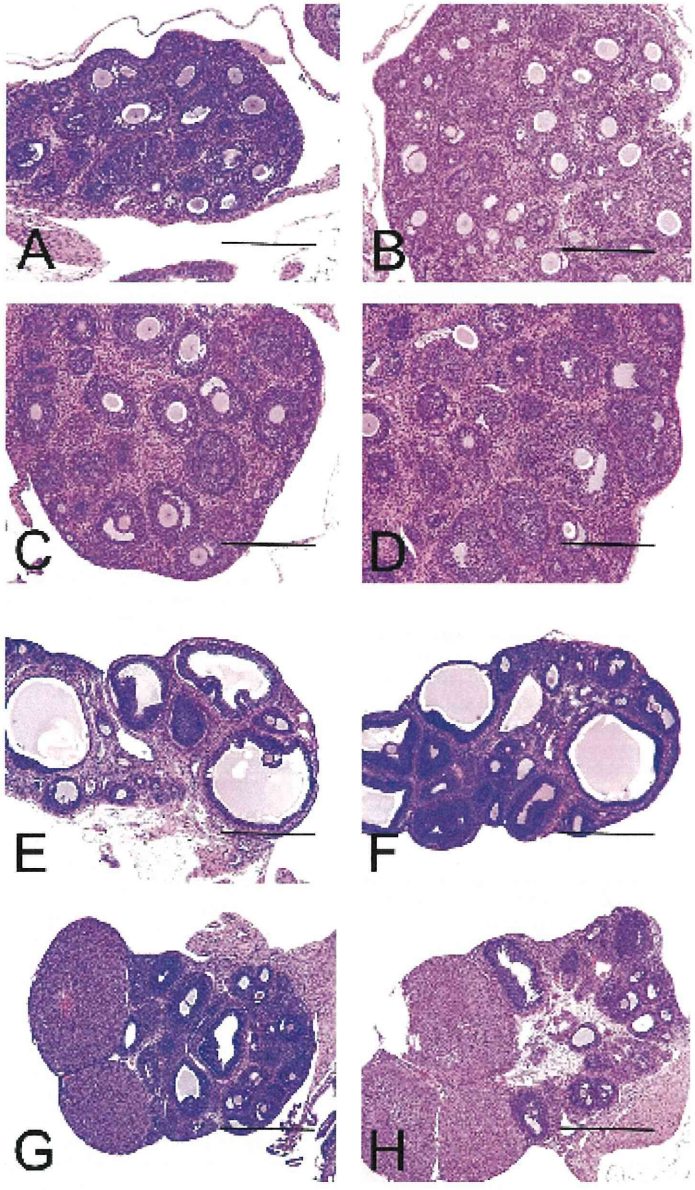

FIGURE 2.—Sequential morphological assessment of the ovaries. A–D and E–H illustrate changes at PND21 and five weeks of age (A, E; B, F; C, G; and D, H are for the 1,500, 150, 15, and 0 μg/kg group, respectively). At five weeks of age, the ovaries in A and B were obtained from animals demonstrating persistent estrus and that were given 1,500 and 150 μg/kg, and the remainder featured estrus with normal cyclicity. Hematoxylin and eosin stain. Scale bars are 1 mm and 2 mm in A–D and E–H, respectively.

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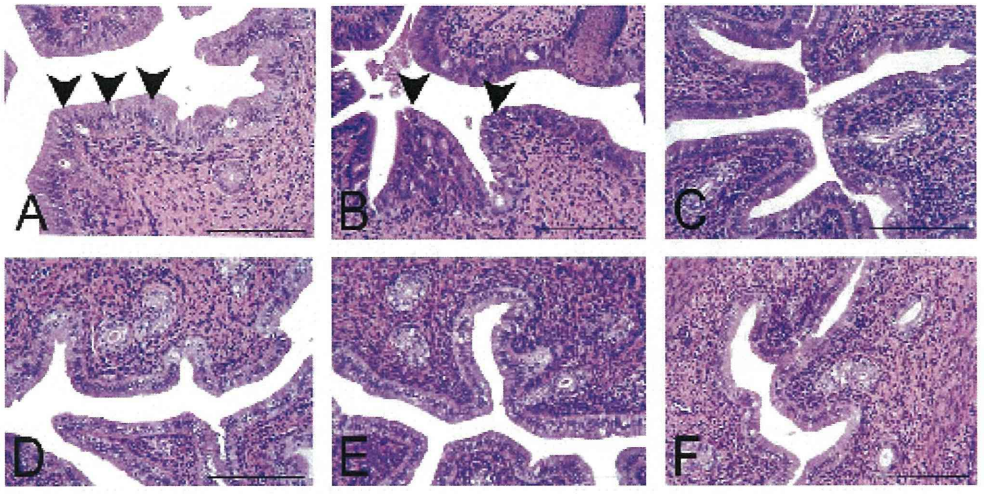

FIGURE 3.—Morphological changes in the uterus at five weeks of age. (A–F) The changes in the 1,500, 150, 15, 1.5, 0.15 and 0 μg/kg group, respectively. The uteri of A and B were obtained from animals demonstrating persistent estrus and that were given 1500 and 150 μg/kg, and the remainder featured estrus with normal cyclicity. (A) Arrowheads indicate high columnar epithelial cells in the luminal surface at 1,500 μg/kg. (B) Arrowheads indicate intraluminal hyperplasia in the luminal epithelium at 150 μg/kg. (C–F) A number of apoptotic cells in the luminal epithelium, which is a typical feature in the uterus at estrus. Hematoxylin and eosin stain. Scale bars are 200 μm.

in the control group. In the other organs, no morphological features were different between treated groups and controls.

Immunohistochemical Intensity of ERα Expression in the Uterus, Vagina, and Ovary

The immunohistochemical expression of ERα in the uterus and vagina is summarized in Figure 5. The intensities were slightly higher in the uterus and vagina at PND14 and PND21 than those at five weeks of age. Estrogen receptor-α did not express in the granulosa cells, theca cells, or stromal cells of the ovary. Treatment-related changes were not detected in any other organs or at any examined time points.

Uterine Carcinogenicity and Histopathology at Termination

The incidences of atypical hyperplasias and endometrial adenocarcinomas are shown in Table 3. Values for both were lower in the highest dose group than in the controls. On the other hand, neoplastic lesions showed an increasing trend in the lower groups, and the incidence of adenocarcinomas and total multiplicity of atypical hyperplasias and adenocarcinomas were significantly higher in the 150 μg/kg group than those in the controls. In the 1.5 and 15 μg/kg groups, which were sacrificed after a shorter period than other groups, few atypical hyperplasias were detected, and the incidence was similar to background data at the same age in this strain.

At termination, morphological abnormalities such as atrophic uterine horns, disappearance of lumina (Figure 6), or short and straight oviducts were detected in the highest dose group. Such abnormalities were not observed in other treated groups. As common changes in all groups including controls, most of the ovaries were atrophic with several cystic atretic follicles and few or no corpora lutea (Figure 6). Several primary follicles remained, with no obvious variation among the control and treated groups. The vaginal mucosa was similarly composed of several layered squamous cells in the control and treated groups. Neoplastic and non-neoplastic lesions were abundant in the treated and control groups, but their incidence and severity were comparable among the groups. Treatment-related morphological changes were not detected in the genital tracts.

mRNA Expression of the Ovary and Uterus at Five Weeks of Age

Data for mRNA expressions of ER α and β, progesterone receptor (PR), and GAPDH in the ovary are shown in Figure 7. Glyceraldehyde 3-phosphate dehydrogenase mRNA was constitutive in all animals. Although no differences in ERα or ERβ could be detected in the ovary or uterus, PR in the uterus demonstrated increased expression at the 150 μg/kg dose.

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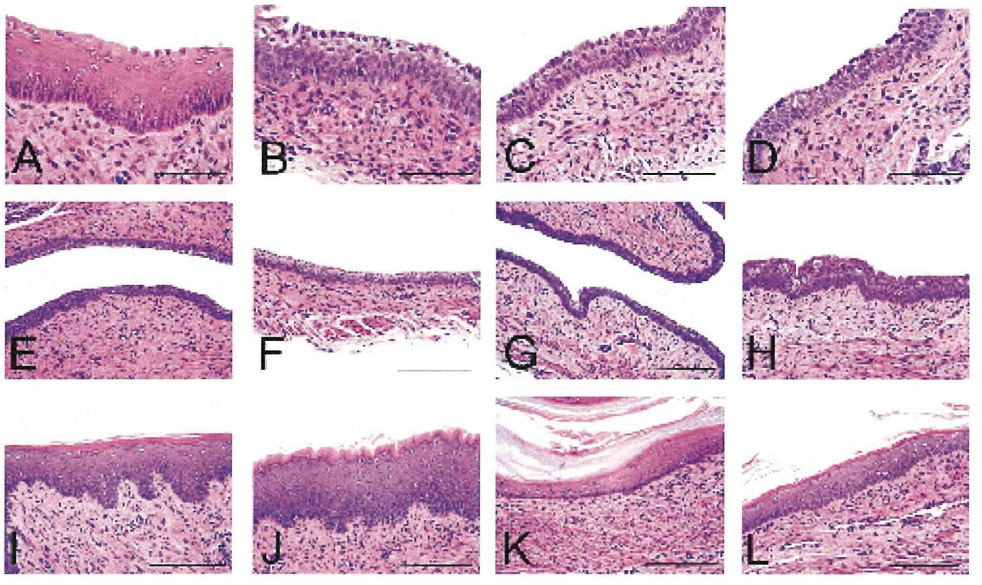

FIGURE 4.—Sequential morphological assessment of the vagina. A–D, E–H, and I–L illustrate changes at PND14, PND21, or five weeks of age (A, E, I; B, F, J; C, G, K, and D, H, L are for the 1,500, 150, 15, and 0 μg/kg groups, respectively). At five weeks of age, the vaginas of A and B were obtained from persistent estrus at 1,500 and 150 μg/kg, and the others were from rats at estrus with normal cyclicity. Hematoxylin and eosin stain. Scale bars are 200 μm and 400 μm in A–D and E–L, respectively.

DISCUSSION

The present study demonstrated clear, dose-dependent, delayed effects of a single neonatal treatment with DES at various doses on morphology in the female reproductive tract and estrous cyclicity in Donryu rats. In particular, earlier onset of PE was evident in all but the lowest dose group. Although timing of VO, which represents puberty, and uterine gland genesis was not affected, the ovarian morphology at five weeks of age at the highest dose indicated an abnormal status with anovulation and excess estrogen levels relative to progesterone (E:P ratio).

In addition, the cornification of vaginal mucosa and uterine intraluminal hyperplasia were consistent with those in rats (Katsuda et al. 2000) or mice (Bern et al. 1987; Yoshida et al. 1999) neonatally exposed to DES or high-dose p-tert octylphenol. The vaginal changes in the present study are considered to be ovary dependent events in rats (Katsuda et al. 2000), but not mice (Forsberg 1979; Takasugi et al. 1962). The present results clearly indicated that the animals at the highest dose were already affected before puberty. Perinatal exposures to high doses of estrogens are known to irreversibly disrupt steroid hormone receptor or related gene expression in the uterus (Nakamura et al. 2008; Yoshida et al. 2000; Yoshida et al. 2002a). The studies on ERα or ERβ mRNA as end points of neonatal exposure to estrogens were limited, whereas PR and AR as well as IGF1 have been shown to be important (Weihua et al. 2002). The present study did not provide proof of the involvement of ERα in both mRNA and protein levels in the uterus. The increased expression in PR in the uterus at 150 μg/kg might be related to fewer corpora lutea, although mechanisms of the increase have not been determined.

Several animals at 150 μg/kg revealed similar PE status and morphology in the female reproductive tract to those in the highest group, indicating that these animals at 150 μg/kg were also affected before puberty. Besides, clear abnormalities including morphological or gene expressions were not detected in the remainder of the 150 μg/kg group and in all rats in the 15 and 1.5 μg/kg groups until five weeks of age. Therefore, the earlier onset of PE in these animals was considered to be a delayed effect. In addition, the onset showed clear dose dependency. The early onset of PE has already been reported as an indicator of DAS (Mobbs et al. 1985), but there are limited data on dose dependency (Bern et al. 1987). Recently, Ninomiya et al. (2007) reported early onset PE with dose dependency in Sprague-Dawley rats exposed to DES at doses of 1–100 μg/rat (approximately equivalent to 17–1,700 μg/kg if the body weights are expected to be 5–6 g at PND0), but not 0.1 μg/rat (approximately equivalent to 1.7 μg/kg) until 150 days of age. Although diversity exists at lower doses, their results showing delayed effect are in line with our data. The

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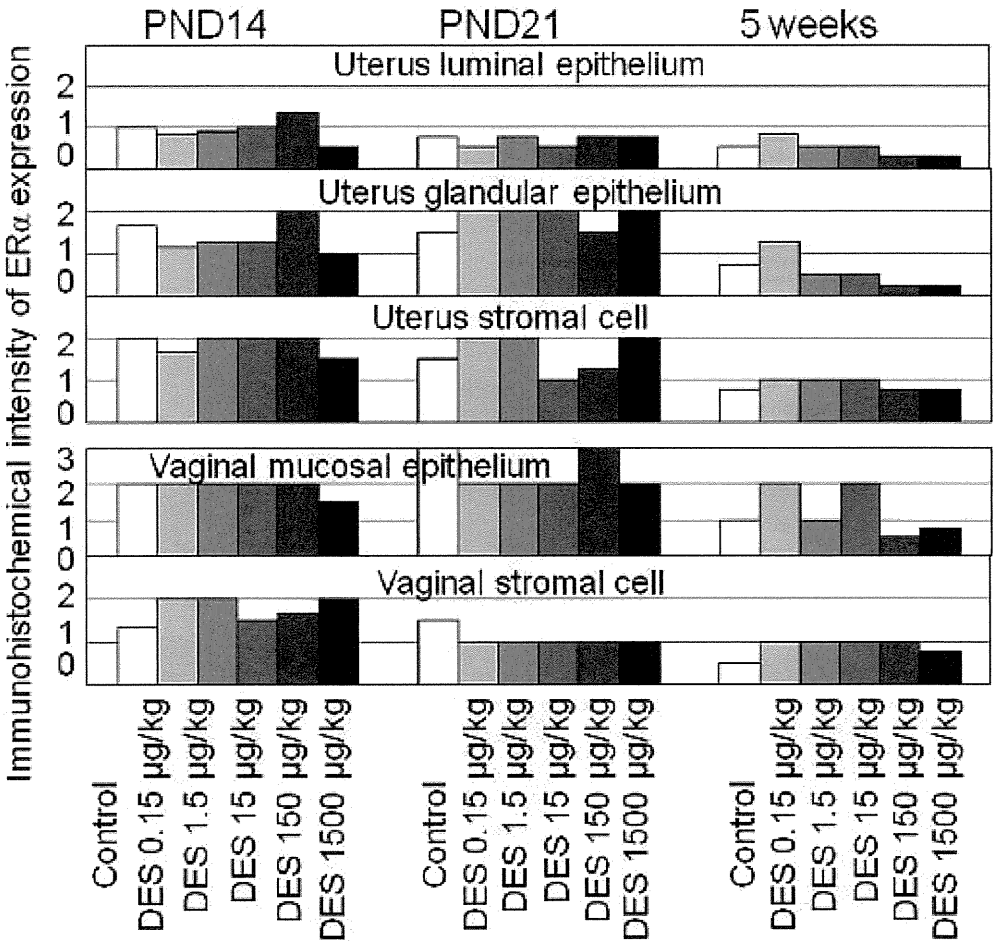

FIGURE 5.—Immunohistochemical intensity of ERα expression in the uterus and vagina at PND14, PND21, and five weeks of age. Treatment-related changes were not detected in any organs. The intensities of these organs at PND14 and PND21 were similar, but overall, expression had declined at five weeks of age.

estrous cyclicity follows a similar tendency until eight months of age in the 0.15 μg/kg and control groups. Although the lower two groups were terminated two months earlier than the other groups, most of the control rats (80%) reached persistent estrus at thirty-three weeks of age. Thus, the lowest dose would not be likely to affect the estrous cycle after eight months of age.

The results of the uterotrophic assay demonstrated that the single treatments with DES at 1.5 μg/kg and higher were inducible doses of estrogenic activity in vivo with dose dependency. Interestingly, our results demonstrated that the doses showing estrogenic activity in vivo corresponded to the inducible doses of early onset of PE. These results suggest that test substances with estrogenic activity in vivo may induce delayed effects. A concern that twenty-four hours after a single treatment is too soon to detect any estrogenic activity in uterotrophic assay might exist, because uterine weights are usually measured twenty-four hours after a three-day treatment with a test substance in a uterotrophic assay (OECD 2007). However, a previous study that demonstrated, using radioautography, that DES di [^{35}S] sulfate injected intraperitoneally at 1 mg/rat

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TABLE 3.—Incidence (%) and multiplicity of uterine proliferative lesions in rats neonatally exposed to diethylstilbestrol.

Group	No. of rats	No lesions	Atypical hyperplasias			Adenocarcinomas	Total incidence of proliferating lesions[a]	Multiplicity[b]
			Slight	Moderate	Severe			
Control	16	35.7	28.6	42.9	7.1	14.3	64.3	1.44 ± 0.53
DES 15 μg/kg	15	0	46.7	53.3	20.0	20.0	100.0	1.60 ± 0.63
DES 150 μg/kg	24	8.4	12.5	54.2	29.2	50.0*	91.6	3.83 ± 0.87*
DES 1500 μg/kg	17	52.9	17.6	23.5	23.5	5.9	47.1	1.29 ± 0.59

Abbreviation: DES, diethylstilbestrol.
* Significantly difference from control value, $p < .05$.
[a] The incidence of rats bearing atypical hyperplasias or adenocarcinomas (%).
[b] The average number of proliferating lesions per rat bearing these lesions.

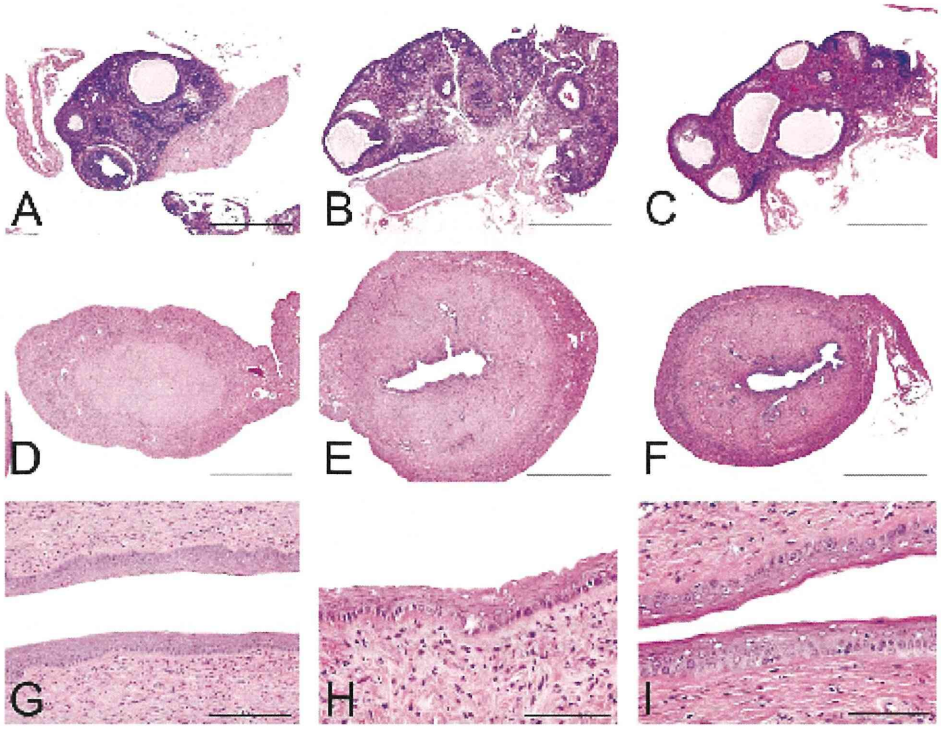

FIGURE 6.—Morphological changes at the termination. A–C, D–F, and G–I are the ovaries, uteri, and vaginas, respectively. (A, D, G) 1,500 μg/kg; (B, E, H) 15 μg/kg; and (C, F, I) 0 μg/kg. Hematoxylin and eosin stain. Scale bars are 1 mm, 2 mm, 400 μm, and 200 μm in A–C, D–F, G, and H–I, respectively.

distributed in the whole body within twenty-four hours (Barford et al. 1977) could be support that the concern could be excluded.

It is well known that neonatal exposure to DES can induce malformations in the female reproductive tract not only rodents (Newbold et al. 1983; Rothschild et al. 1987–88), but also in

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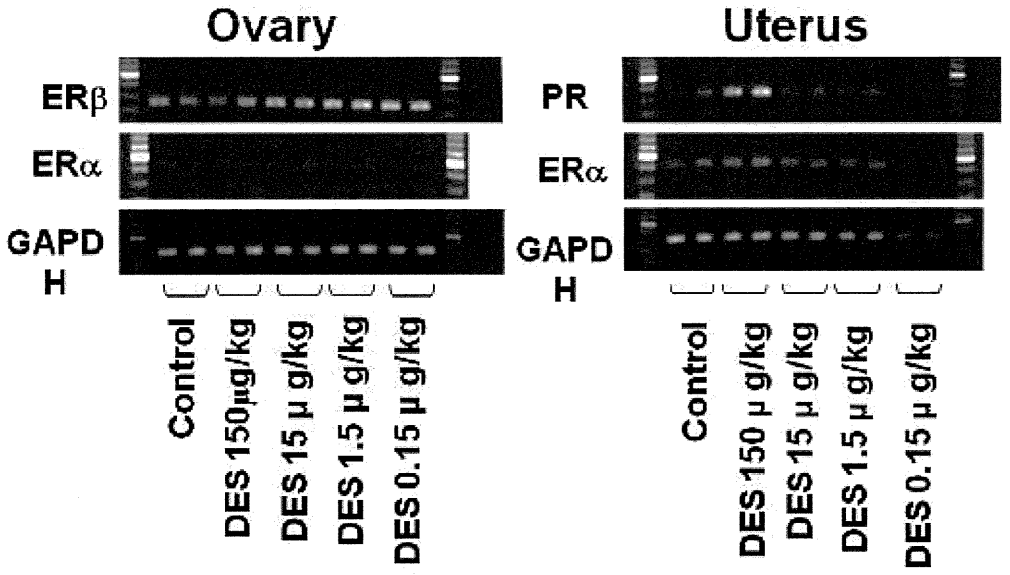

FIGURE 7.—mRNA Expression of estrogen receptors α and β and progesterone receptor in the ovary and uterus in the 150, 15, 1.5, 0.15 and 1.5 μg/kg and control groups at five weeks of age. The ovaries and uteri at 150 μg/kg were obtained from animals in persistent estrus, and others are from rats at estrus with normal cyclicity.

human beings as DES daughters (Jeffries et al. 1984). The present results of uterine carcinogenesis did not show a dose dependency. The reason is likely to be related to the extreme atrophy without the endometrial epithelium apparent in the uteri at the highest dose at termination, because the epithelial cells are the histogenesis of endometrial adenocarinomas. The increase of endometrial adenocarcinoma development at 150 μg/kg of DES observed here is in line with previous studies of neonatal treatment with estrogenic compounds such as DES, tamoxifen, or high-dose p-tert octylphenol (Carthew et al. 2000; Newbold et al. 1990; Yoshida et al. 2002b). Prolonged exposure to excess estrogen relative to progesterone in the blood (increased E:P ratio), which is reflected as PE in a vaginal smear, is an important cause of uterine cancer development in Donryu rats, a high-yield strain of uterine endometrial cancers (Ando-Lu et al. 1994; Nagaoka et al. 1990; Vollmer 2003; Yoshida et al. 2004). Excess estrogens also play a crucial role in cancer of the uterine corpus in other strains of rats (Deerberg and Kaspareit 1987) or mice, as well as women (World Health Organization Classification of Tumours 2003), although androgen or prolactin might influence uterine cancer development in rodents (Nantermet et al. 2005; Yoshida et al. 2009). Our results described above show that early onset of PE might be the most sensitive indicator of delayed effects by neonatal exposure to estrogenic compounds rather than susceptibility to induction of preneoplastic and neoplastic lesions and other morphological changes.

It is still unclear what mechanism is crucial for the early onset of PE. MacLusky and Naftolin (1981) first reported such effects as DAS, and they hypothesized that disruption of the hypothalamus might be the trigger. There is evidence of involvement of loss of noradrenergic neuronal responsiveness in rats (Lookingland and Barraclough 1982) and that DAS is caused by age-related neuroendocrine function rather than ovarian impairment (Mobbs et al. 1985). Recently, Adewale et al. (2009) reported gonadotropin-releasing hormone (GnRH) release following neonatal estrogen exposure to be normal in the organum vasculosum of the lamina terminals in rats displaying early onset of irregular cycles, including PE. One present focus is on the newly discovered neuropeptide in the hypothalamus, kisspeptin, a regulator of gonadtropin secretion via GnRH secretion (Messager et al. 2005), because it plays pivotal roles in the onset of puberty (de Roux et al. 2003; Seminara et al. 2003), sex differentiation (Kauffinan et al. 2007), and reproductive activities such as estrous cycling (Adachi et al. 2007) and ovulation (Clarkson and Herbison 2009; Uenoyama et al. 2009). It has been established that neonatal exposure to estrogens or estrogenic compounds decreased hypothalamic Kiss 1 mRNA levels or stimulation of GnRH neurons by kisspeptins, resulting in

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impairment of female reproductive functions (Bateman and Patisaul 2008; Navarro et al. 2009). Kisspeptin and its signaling pathway in GnRH neurons might be anticipated as key factors in delayed effects.

In conclusion, neonatal exposure to a single dose of DES, which shows estrogenic activity in vivo, induces delayed adverse effects on female rats in a dose-dependent manner. Detection of early onset of PE by vaginal smear appears to be the most sensitive and useful parameter to detect such effects, though a wait of over four months is necessary for significance to become apparent. Its appearance, however, can be considered adverse in rats as well as human beings, because it is irreversible and increases the risk to development of uterine corpus cancer.

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Differential Stimulation Pathways of Progesterone Secretion from Newly Formed Corpora Lutea in Rats Treated with Ethylene Glycol Monomethyl Ether, Sulpiride, or Atrazine

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Ethylene glycol monomethyl ether (EGME), sulpiride, and atrazine are known ovarian toxicants, which increase progesterone (P4) secretion and induce luteal cell hypertrophy following repeated administration. The aim of this study was to define the pathways by which these compounds exerted their effects on the ovary and hypothalamic-pituitary-gonadal (HPG) axis. In the ovary, changes in the steroidogenic activity of new and old corpora lutea (CL) were addressed. EGME (300 mg/kg), sulpiride (100 mg/kg), or atrazine (300 mg/kg) were orally given daily for four times from proestrus to diestrus in normal cycling rats. Treatment with all chemicals significantly increased serum P4 levels, and EGME as well as sulpiride induced increases in prolactin (PRL) levels. In new CL, at both the gene and the protein levels, all three chemicals upregulated the following steroidogenic factors: scavenger receptor class B type I, steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage, and 3β-hydroxysteroid dehydrogenase (HSD) and downregulated the luteolytic gene, *20α-HSD*. Coadministration of EGME and bromocriptine, a D2 agonist, completely inhibited PRL but not P4 secretion. Additionally, steroidogenic factor expression levels were upregulated, and *20α-HSD* level was downregulated in new CL. These results suggest that EGME both directly and indirectly stimulates P4 production in luteal cells, whereas sulpiride elevates P4 through activation of PRL secretion in the pituitary. Atrazine may directly activate new CL by stimulating steroidogenic factor expressions. The present study suggests that multiple pathways mediate the effects of EGME, sulpiride, and atrazine on the HPG axis and luteal P4 production in female rats *in vivo*.

Key Words: **progesterone; newly formed corpora lutea; ethylene glycol monomethyl ether; sulpiride; atrazine; laser microdissection.**

Numerous drugs and chemicals have been found to interfere with reproductive function in female experimental animals (Yuan and Foley, 2002). The female reproductive organs are regulated across the estrous cycle by the hypothalamic-pituitary-gonadal (HPG) system through a complex feedback loops. These feedback mechanisms are perturbed by ovarian toxicants including ethylene glycol monomethyl ether (EGME), sulpiride, and atrazine. In rodents, repeated exposure to ovarian toxicants produces identifiable histopathological changes in the reproductive tract as well as abnormal hormone secretion and disrupted estrous cycles (Sanbuissho *et al.*, 2009).

EGME, sulpiride, and atrazine induce luteal hypertrophy following repeated administration (Davis *et al.*, 1997; Dodo *et al.*, 2009; Shibayama *et al.*, 2009; Yuan and Foley, 2002). EGME, which is widely used in various industrial products such as detergents, adversely affects both the male and the female reproductive systems (Johanson, 2000; Welsch, 2005). In females, EGME and its active metabolite, 2-methoxy acetic acid, induce the hypersecretion of progesterone (P4) from luteal cells both *in vivo* and *in vitro* (Almekinder *et al.*, 1997; Davis *et al.*, 1997). Sulpiride, a dopamine D2 antagonist, is used clinically as an atypical antipsychotic drug (Lacruz *et al.*, 2000). In rats, D2 antagonists block the inhibitory effect of dopamine on prolactin (PRL) release, which results in the preservation of functional corpora lutea (CL) and produces a pseudopregnant state (Rehm *et al.*, 2007). Atrazine, a chlorotriazine herbicide, is a potent endocrine disruptor, which promotes mammary tumor growth in female rats (Eldridge *et al.*, 1994) and alters the central nervous system regulation of the reproductive system in mammals (Eldridge *et al.*, 1999). Recently, atrazine has been shown to inhibit the release of gonadotropin-releasing hormone and diminish the estrogen-induced surge of luteinizing hormone (LH) and PRL in rats (Cooper *et al.*, 2000, 2007). Other studies indicate that atrazine also stimulates steroidogenesis and increases serum concentrations of P4 and corticosterone in female rats (Fraites *et al.*, 2009). Effects of atrazine on the HPG

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axis have been previously reported; however, its direct effects on the ovary are not completely understood.

In normal rat ovaries, CL are formed and regress over several estrous cycles. Consequently, at any one time, the rat ovary will contain CL at various stages of development and regression. The CL are classified into two main types: those which are newly formed by the current ovulation (new CL) and CL remaining from prior estrous cycles (old CL) (Bowen and Keyes, 2000). Each type is morphologically distinguishable at each estrous stage (Yoshida *et al.*, 2009). A number of key factors within the HPG axis are related to P4 biosynthesis in the CL. Scavenger receptor class B type I (SR-BI), steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage (P450scc), and 3β-hydroxysteroid dehydrogenase (HSD) are critical for the uptake, synthesis, and transport of cholesterol and the processing of cholesterol to P4 in luteal cells (Stocco, 2001; Stocco *et al.*, 2007). P4 secretion from the CL in rodents is regulated by the balance between synthesis and catabolism. Briefly, it depends not only on the amount of P4 synthesized but also on the expression of the enzyme 20α-HSD that catabolizes P4 into the inactive progestin, 20α-dihydroprogesterone (20α-DHP). Once 20α-HSD is expressed in the CL, P4 secretion declines and 20α-DHP becomes the major steroid secreted by luteal cells (Stocco *et al.*, 2000). This process is equivalent to the functional regression of the CL. Thus, changes in 20α-HSD activity and the level of its inducible prostaglandin F2α (PGF2α) are considered to be the important factors of luteolysis (Stocco *et al.*, 2007). This pathway may also be the target of agents, which block luteal regression, including the previously mentioned ovarian toxicants.

EGME, sulpiride, and atrazine all target luteal cells with a resulting increase in serum P4; because their chemical structure and properties are completely different, it is speculated that their toxicological mechanisms in CL were different among them, but their mechanisms *in vivo* have not been elucidated. The present study was performed to clarify how these agents target the luteal cells and affect the HPG axis *in vivo*. This study focused on changes in the gene expression levels of steroidogenic factors (*SR-BI*, *StAR*, *P450scc*, and *3β-HSD*) and luteolytic factors (*20α-HSD* and *PGF2α receptor* [*PGF2α-R*]) in new and old CL, which were isolated with laser microdissection (LMD). In addition, serum hormone assays and a morphometric analysis of immunohistochemical staining intensities of steroidogenic factors were also performed. For EGME, its direct luteal effect was examined under a PRL inhibitory state by coadministration of EGME and the D2 agonist bromocriptine (BRC).

MATERIALS AND METHODS

Animals. Female 6-week-old Sprague-Dawley (CrlCD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were maintained at 23–25°C and a relative humidity of 50–60% with a 12 h light cycle. Commercial rodent chow (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan) and drinking water were available *ad libitum*. Estrous cycle stage was determined each morning by vaginal smear. Only animals displaying 4-day estrous cycles were included in the experiments. The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Chemicals and reagents. EGME was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and was dissolved in water for injection. Sulpiride and atrazine were purchased from Wako Pure Chemical Industries, Ltd and Tokyo Chemical Industry (Tokyo, Japan), respectively, and were suspended in 0.5% w/vol methyl cellulose 400 solution (Wako Pure Chemical Industries, Ltd). BRC was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in 70% ethanol diluted with saline. Control animals received vehicle solutions at each experiment.

Experiment 1. To confirm the histopathological changes of luteal cell hypertrophy following treatment with EGME, sulpiride, and atrazine, the animals were gavaged once per day for 2 weeks (Supplementary fig. 1A). The doses used for the study were as follows: EGME (300 mg/kg), sulpiride (100 mg/kg), and atrazine (300 mg/kg). Effective doses on the ovary and dosing period were selected based on the relevant literatures (Davis *et al.*, 1997; Dodo *et al.*, 2009; Ishii *et al.*, 2009; Shibayama *et al.*, 2009). After treatment, the animals were euthanized in the morning ($n = 5$–8 per group). The ovary and other reproductive and endocrine organs (uterus, vagina, pituitary, adrenal, thyroid, and mammary gland) were fixed in 4% paraformaldehyde and 10% vol neutral-buffered formalin, respectively, and routinely processed with hematoxylin and eosin (HE) staining.

Experiment 2. Because the repeated administration of EGME, sulpiride, or atrazine would disrupt the estrous cycle within 1 week, short-term treatment was required for clearly distinguishing the luteal effects of these chemicals on new CL from those on old CL in normal cycling animals. The animals were treated with these chemicals by gavage for four times from proestrus to diestrus, once per day in the morning (Supplementary fig. 1B). The doses were the same as for experiment 1. Between 1000 and 1100 h of the fourth day of treatment, when all animals were in diestrus, they were euthanized by decapitation after 4 h (EGME and sulpiride) or 1 h (atrazine) following the last treatment ($n = 4$–7 per group). The necropsy time points were chosen based on previously reported pharmacokinetic data for these agents (Praiten *et al.*, 2009; Hays *et al.*, 2000; Lawn *et al.*, 2009; McMullin *et al.*, 2007; Yamada *et al.*, 1990). The serum was stored at −20°C until use in the hormone assays. For LMD, the left ovaries were rapidly removed, embedded in optimal cutting temperature (OCT) compound, and frozen with liquid nitrogen. The right ovaries were fixed in 4% paraformaldehyde and serially sectioned. Sections were then stained with HE or used for immunohistochemical studies.

Experiment 3. To evaluate the luteal effects of EGME in the absence of a PRL effect, the animals were treated with EGME (300 mg/kg) by gavage, BRC (2 mg/kg) by sc injection, or EGME and BRC once per day from proestrus to diestrus as the same procedure of experiment 2 (Supplementary fig. 1C). Effective dose of BRC was selected based on the relevant literature (Bridges and Ronsheim, 1990). On the fourth day of treatment, when all animals were in diestrus, they were euthanized by decapitation between 1000 and 1100 h after 4 h following the last treatment ($n = 4$–5 per group). The serum hormone assays, gene expression analysis of CL with LMD, and immunohistochemical examination of CL were performed in the same manner as for experiment 2, omitting fixation of ovary in 10% vol neutral-buffered formalin instead of 4% paraformaldehyde.

Hormone assays. The serum concentrations of P4, estradiol-17β (E_2), PRL, LH, and follicle-stimulating hormone (FSH) were determined using double-antibody radioimmunoassay and ^{125}I-labeled radioligands. P4 and E_2 were measured as described by Taya *et al.* (1985). National Institute of Diabetes and Digestive and Kidney Disease radioimmunoassay kits were employed for rat PRL, LH, and FSH (National Institute of Arthritis, Metabolism and Digestive Diseases [NIAMDD], National Institutes of Health [NIH], Bethesda, MD) (Taya *et al.*, 1983).

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LMD of new and old CL. The OCT-embedded frozen ovaries were sectioned onto membrane-based LMD slides (Leica Microsystems, Wetzlar, Germany) and fixed in 75% ethanol. The sections were then stained with 1% toluidine blue solution, dehydrated, and air-dried. New CL (CL formed by the current ovulation) and old CL (CL remaining from prior estrous cycles) were captured using a Leica LMD6000 LMD system (Leica Microsystems).

Extraction of total RNA and reverse transcription. Laser-captured tissues were pooled in lysis buffer, and RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Residual genomic DNA was removed with an RNase-free DNase Set (Qiagen). The concentration and purity of RNA were checked using a spectrophotometer (NanoDrop ND-1000, Thermo Fischer Scientific Inc., Waltham, MA) and stored at −80°C until analysis. For complementary DNA synthesis, reverse transcription (RT) was performed with the Sensiscript RT Kit (Qiagen) following the manufacturer's instructions.

Real-time quantitative PCR. Messenger RNA (mRNA) levels of *SR-BI*, *StAR*, *P450scc*, *3β-HSD*, *20α-HSD*, *PGF2α-R*, *PRL receptor* (*PRL-R*) (*Long* and *Short forms*), *steroidogenic factor 1* (*SF-1*), *nuclear receptor 5A2* (*NR5A2*), *acetyl-coenzyme A acetyltransferase 1* (*ACAT-1*), and *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) (Table 1) were analyzed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The probes and primers of *SF-1* (Rn00584298_m1) and *ACAT-1* (Rn00579605_m1) were prepared with TaqMan gene expression assays (Applied Biosystems). To compare mRNA levels among samples, mRNA levels for each gene were normalized to a housekeeping gene, *HPRT*, using the standard curve method.

Immunohistochemistry. The ovarian sections were deparaffinized, treated with 90% methanol containing 3% H_2O_2 for 10 min at room temperature, heated in a 0.01M citric acid buffer (pH = 7.0) at 95°C for 5 min, and incubated with antibodies of SR-BI (NB400-104; Novus Biologicals, Littleton, CO), StAR (sc-25806; Santa Cruz, CA), and P450scc (AB1244; Millipore Corporation, Temecula, CA) overnight at 4°C. For 3β-HSD (sc-30821; Santa Cruz) immunostaining, there was no antigen retrieval treatment in experiment 2; heating in a citric acid buffer at 95°C for 10 min was performed in experiment 3. The sections were then incubated with the secondary antibody (HISTOFINE SIMPLESTAIN MAX-PO, Nichirei Bioscience, Tokyo, Japan) and were visualized with 3,3′-diaminobenzidine and counterstaining with hematoxylin.

Morphometric analysis of immunohistochemical staining intensity. Immunohistochemical staining intensities of SR-BI, StAR, P450scc, and 3β-HSD in new and old CL were quantified using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). Pictures obtained from different areas (five areas per animal) of new and old CL ($n = 4$–5) were taken using a ×40 objective and a digital camera system. Digital images were processed by subtracting the light background, splitting the blue channel to remove the hematoxylin staining, followed by adjustment of the density threshold (SR-BI, StAR, and P450scc; 0–210, 3β-HSD; 0–220). The pixel intensity was measured and compared by calculating the integrated density, which was the product of the area and the mean gray value.

TABLE 1
Primers and Probes Used for Real-Time PCR Analysis

Gene	Primer and probe		GenBank accession No.
SR-BI	Forward	5′-CCGAATCCTCACTGGAATTCTTC-3′	NM_031541
	Reverse	5′-CGAACACCCTTGATTCCTGGTA-3′	
	Probe	5′-VIC-AAGCCTGCAGATCTATGA-MGB-3′	
StAR	Forward	5′-GGGAGAGTGGAACCCAAATGT-3′	NM_031558
	Reverse	5′-CATGGGTGATGACTGTGTCTTTTC-3′	
	Probe	5′-VIC-AAGGAAATCAAGGTCCTGAAG-MGB-3′	
P450scc	Forward	5′-TCTCCTCTACCAACAGTCCTCGAT-3′	BC089100
	Reverse	5′-TGGTACAGGTTTATCCAACCATTG-3′	
	Probe	5′-VIC-CTTCAATGAGATCCCTTC-MGB-3′	
3β-HSD	Forward	5′-GCCCAACTCCTACAAGAAGATCAT-3′	L17138
	Reverse	5′-CTCGGCCATCTTTTTGCTGTATG-3′	
	Probe	5′-VIC-ATGTGCTTTCATGATGCTCT-MGB-3′	
20α-HSD	Forward	5′-TTTCAATGAGGAGAGAATCAGAGAGA-3′	L32601
	Reverse	5′-CCATGTCATCTGAAGCCAACTG-3′	
	Probe	5′-VIC-CCTGCAGGTCTTTGAT-MGB-3′	
PGF2α-R	Forward	5′-CTCTGGCTTGTGCCCACTTT-3′	U47287
	Reverse	5′-CCGATGCACCTCTCAATGG-3′	
	Probe	5′-VIC-CCTGGGCAGTACGATG-MGB-3′	
PRL-R (Long form)	Forward	5′-CTGGGCAGTGGCTTTGAAG-3′	M57668
	Reverse	5′-CCAAGGCACTCAGCAGCTCT-3′	NM_012630
	Probe	5′-FAM-ATCTTTCCACCAGTTCCTGGGCCAAAAATA-MGB-3′	
PRL-R (Short form)	Forward	5′-CTGGGCAGTGGCTTTGAAG-3′	M57668
	Reverse	5′-AAGGGCCAGGTACAGATCCA-3′	NM_012630
	Probe	5′-FAM-ATCTTTCCACCAGTTCCTGGGCCAAAAATA-MGB-3′	
NR5A2	Forward	5′-TTCGGGCCAATGTACAAGAGA-3′	NM_021742
	Reverse	5′-GGCTCGAATGAGGGCTTTCT-3′	
	Probe	5′-VIC-ACAGGGCCTTGAAAC-MGB-3′	
HPRT	Forward	5′-GCCGACCGGTTCTGTCAT-3′	X62085
	Reverse	5′-GGTCATAACCTGGTTCATCATCAC-3′	
	Probe	5′-FAM-CAGTCCCAGCGTCGTG-TAMRA-3′	

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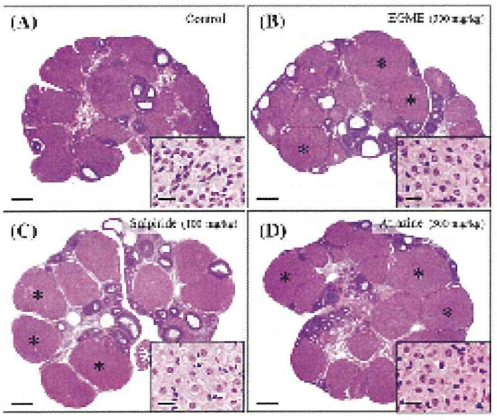

FIG. 1. Luteal cell hypertrophy induced by the 2-week administration of EGME, sulpiride, or atrazine. Luteal cells become hypertrophied with abundant eosinophilic cytoplasm following EGME (B), sulpiride (C), or atrazine (D) treatment compared with respective control CL at the diestrus stage (A). Asterisks indicate hypertrophied CL. Bars represent 500 μm, and bars in the inset images represent 20 μm.

Statistical analysis. All data are presented as the mean ± SEM. Differences in hormone assays, mRNA level in new and old CL, and immunohistochemical staining intensity were evaluated by Student's or Welch's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Experiment 1

Luteal cell hypertrophy induced by a 2-week treatment of EGME, sulpiride, or atrazine. The animals treated with EGME, sulpiride, or atrazine showed abnormal estrous cycle including persistent diestrus as determined by vaginal cytology within 2 weeks. Luteal cell hypertrophy and an increased number of large, atretic follicles were observed on histology in all treatment groups (Figs. 1B–D). New CL were rarely detected. The hypertrophic CL contained round to polygonal luteal cells with abundant eosinophilic and sometimes well-vacuolated cytoplasm. Few apoptotic cells were observed. In the control females, the ovaries at diestrus contained new CL characterized by foamy basophilic luteal cells and old CL with some vacuolated eosinophilic luteal cells and numerous fibrous cells (Fig. 1A). With respect to other reproductive and endocrine organs, mucinous degeneration of the vagina occurred in all treatment groups, and hyperplasia of the mammary gland was found in all sulpiride-treated and some EGME-treated animals, being most pronounced in the former group (data not shown).

Experiment 2

Alterations of serum hormone concentrations. The serum P4 level was significantly higher in all treatment groups compared with the respective control groups at diestrus (2.1-, 4.2-, and 6.3-fold in EGME, sulpiride, and atrazine groups, respectively) (Fig. 2A). In the EGME- and sulpiride-treated groups, the PRL level was significantly higher (4.3- and 12.1-fold in EGME and sulpiride groups, respectively) (Fig. 2B). Other hormone levels (E_2, LH, and FSH) were within basal levels and not changed in all groups (data not shown).

Changes of steroidogenic and luteolytic gene expression levels in new and old CL. New CL of the EGME and sulpiride treatment groups showed significantly higher expression levels of *SR-BI*, *StAR*, *P450scc*, and *3β-HSD* mRNA ($p < 0.01$ or 0.05) and lower levels of *20α-HSD* mRNA ($p < 0.05$) than controls at diestrus (Figs. 3A–E). The *PGF2α-R* mRNA level in new CL of the EGME group was also lower than in the control (Fig. 3F). Atrazine-treated new CL showed a significantly higher level of *SR-BI* mRNA ($p < 0.05$). Of the other steroidogenic factors, *StAR*, *P450scc*, and *3β-HSD* had higher mRNA expression levels and the luteolytic factor, *20α-HSD*, had a lower mRNA level (Figs. 3A–E). In the old CL, the *3β-HSD* and *PGF2α-R* mRNA levels were significantly increased by EGME treatment ($p < 0.05$) (Fig. 3D).

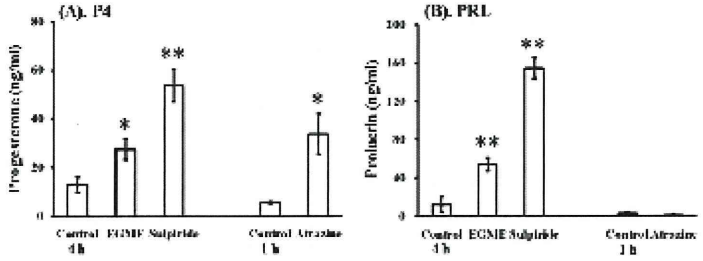

FIG. 2. Serum hormone levels following four daily treatments with EGME, sulpiride, or atrazine. Data represent serum P4 (A) and PRL (B) levels (mean ± SEM). Animals were euthanized at 4 h (EGME and sulpiride) or 1 h (atrazine) after the last dose ($n = 4$–5). Double asterisks ($p < 0.01$) and asterisk ($p < 0.05$) indicate significant differences as compared with the controls.

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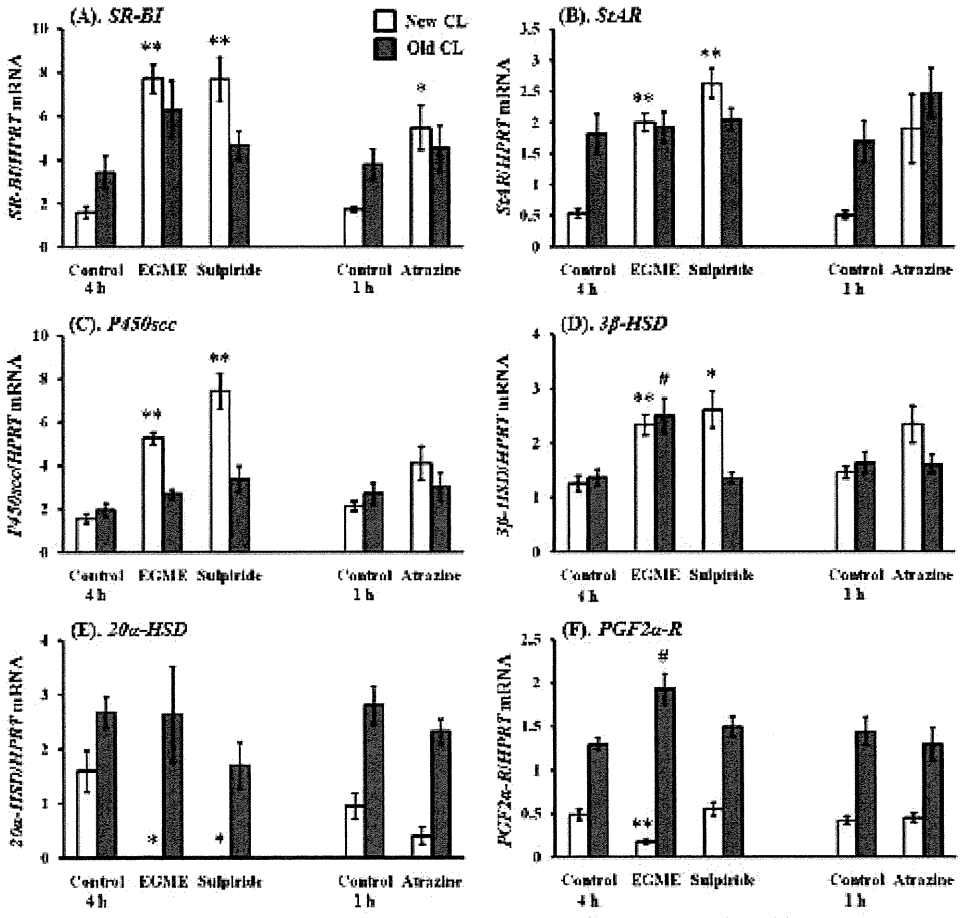

FIG. 3. Effects of EGME, sulpiride, or atrazine on gene expression levels of steroidogenic and luteolytic factors in new and old CL. Relative mRNA levels of *SR-BI* (A), *StAR* (B), *P450scc* (C), *3β-HSD* (D), *20α-HSD* (E), and *PGF2α-R* (F) are presented. Animals were euthanized at 4 h (EGME and sulpiride) or 1 h (atrazine) after the last dose ($n = 4$–5). Data are normalized for *HPRT* mRNA levels in each sample and presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, #$p < 0.05$).

Of the other genes related to steroidogenesis, both types of *PRL-R* (*Long* and *Short forms*) genes were significantly increased in new and old CL of the EGME group and new CL of the sulpiride group (Figs. 4A and 4B). The most drastic change was an increased *NR5A2* mRNA level in new CL of the EGME (8.7-fold) and sulpiride (13.4-fold) groups (Fig. 4D). The *ACAT-1* mRNA levels in new CL of the EGME and sulpiride groups were significantly higher than those of the control group (Fig. 4E). In the atrazine-treated CL, there was a trend to higher mRNA levels of *SF-1* and *ACAT-1* mRNA in the new CL; however, this was not significant (Figs. 4C and 4E).

Morphometric analysis of the immunohistochemical staining of steroidogenic factors in new and old CL. There were no histopathological changes in the HE-stained specimens in any of the treatment groups compared with the controls following daily four times of treatment (data not shown). In the analysis of the SR-BI-, StAR-, P450scc-, and 3β-HSD-positive luteal cells in new and old CL (Fig. 5 and Supplementary figs. 2 and 3), the

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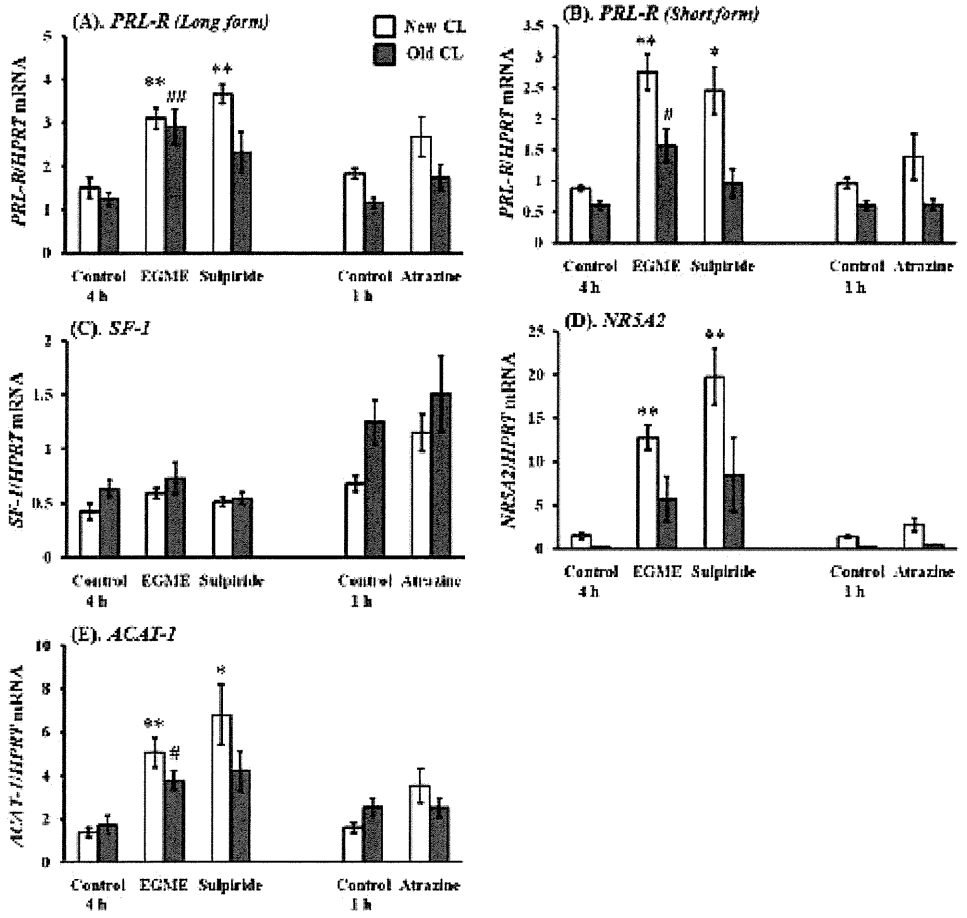

FIG. 4. Effects of EGME, sulpiride, or atrazine on gene expression levels of *PRL-R (Long form)* (A), *PRL-R (Short form)* (B), *SF-1* (C), *NR5A2* (D), and *ACAT-1* (E) in new and old CL. Animals were euthanized at 4 h (EGME and sulpiride) or 1 h (atrazine) after the last dose ($n = 4$–5). Data were normalized for *HPRT* mRNA levels in each sample and presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, ##$p < 0.01$, #$p < 0.05$).

integrated density was mostly higher in new CL than in old CL. New CL in all treatment groups showed significantly higher intensities of these four steroidogenic factors. In comparison to the respective controls, SR-BI and StAR were significantly higher in all treatment groups (up to 2.0- and 6.1-fold in SR-BI and StAR, respectively), P450scc in the EGME and sulpiride groups (up to 1.9-fold), and 3β-HSD in the EGME and atrazine groups (up to 1.6-fold) (Figs. 5A–D). Intensities of the four steroidogenic factors in old CL also showed a higher tendency in all treatment groups and were significantly higher for SR-BI in the atrazine group (1.5-fold), P450scc in the EGME and sulpiride groups (1.9-fold), and 3β-HSD in the EGME group (1.8-fold) in comparison to the controls (Figs. 5A, 5C, and 5D).

Experiment 3

Alterations of serum hormone concentrations. Serum P4 levels were significantly higher in the EGME (2.7-fold) and EGME + BRC (2.0-fold) groups compared with those in the control groups (Fig. 6A). The PRL level was significantly higher in the EGME group (2.7-fold) and tended to be lower

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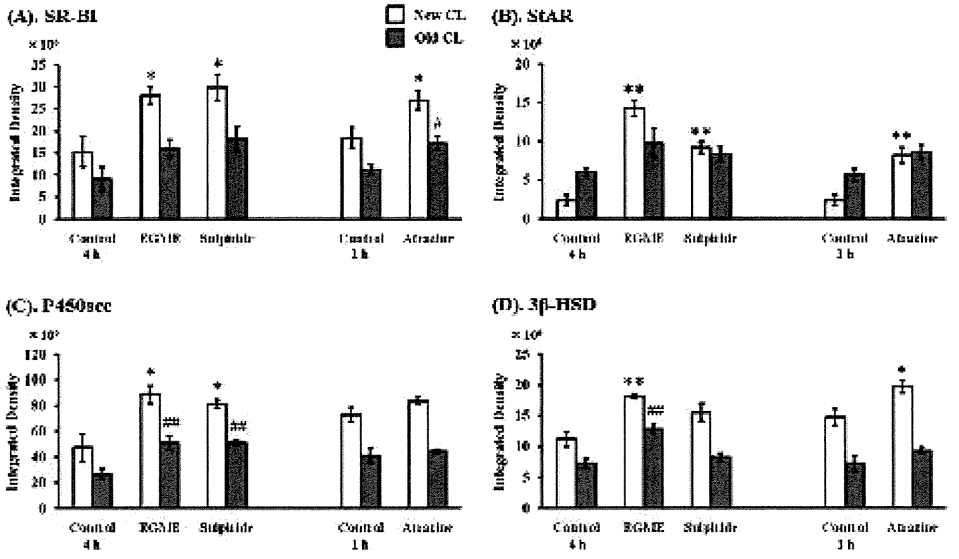

FIG. 5. Effects of EGME, sulpiride, or atrazine on immunoreactivities of steroidogenic factors in new and old CL. Integrated densities of SR-BI (A), StAR (B), P450scc (C), and 3β-HSD (D) are presented. Animals were euthanized at 4 h (EGME and sulpiride) or 1 h (atrazine) after the last dose ($n = 4$–5). Data were presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, ##$p < 0.01$, #$p < 0.05$).

in the BRC (0.1-fold) and EGME + BRC (0.05-fold) groups in comparison to the controls (Fig. 6B). No significant difference was observed in E_2, LH, and FSH levels (data not shown).

Changes of steroidogenic and luteolytic gene expressions in new and old CL. In new CL of the EGME and EGME + BRC groups, mRNA expression levels of steroidogenic factors were significantly higher ($p < 0.01$ or 0.05) and those of luteolytic factors were significantly lower ($p < 0.01$) than in the control group, though the effect was less pronounced with combined BRC treatment (Figs. 7A–F). In contrast, BRC treatment alone significantly increased the *20α-HSD* mRNA level in new CL ($p < 0.01$) (Fig. 7E). In old CL, mRNA levels of *SR-BI* in the EGME and EGME + BRC groups, *P450scc* and *PGF2α-R* in the EGME group, and *3β-HSD* in the EGME + BRC group were significantly increased ($p < 0.01$ or 0.05) (Figs. 7A, 7C, 7D, and 7F).

For the other steroidogenesis-related genes, both forms of *PRL-R*, *NR5A2*, and *ACAT-1* mRNA levels were significantly increased in new and old CL in the EGME group and in new CL of the EGME + BRC group ($p < 0.01$ or 0.05) (Figs. 8A–D). Cotreatment of EGME and BRC also increased *PRL-R* (*Long form*) mRNA in old CL (Fig. 8A). No significant changes in these genes were observed in the BRC group.

Morphometric analysis of the immunohistochemical staining of steroidogenic factors in new and old CL. There were no

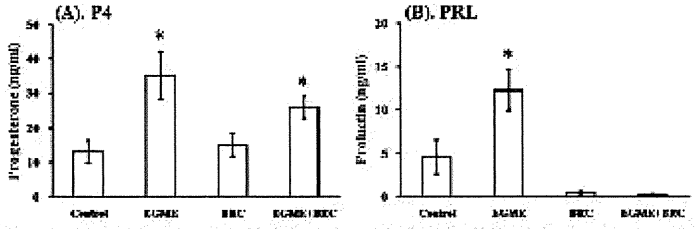

FIG. 6. Serum hormone levels after four daily treatments of EGME, BRC, or EGME and BRC. Data represent serum P4 (A) and PRL (B) levels (mean ± SEM). Animals were euthanized 4 h after the last dose ($n = 4$–5). Asterisk ($p < 0.05$) indicates significant differences as compared with the controls.

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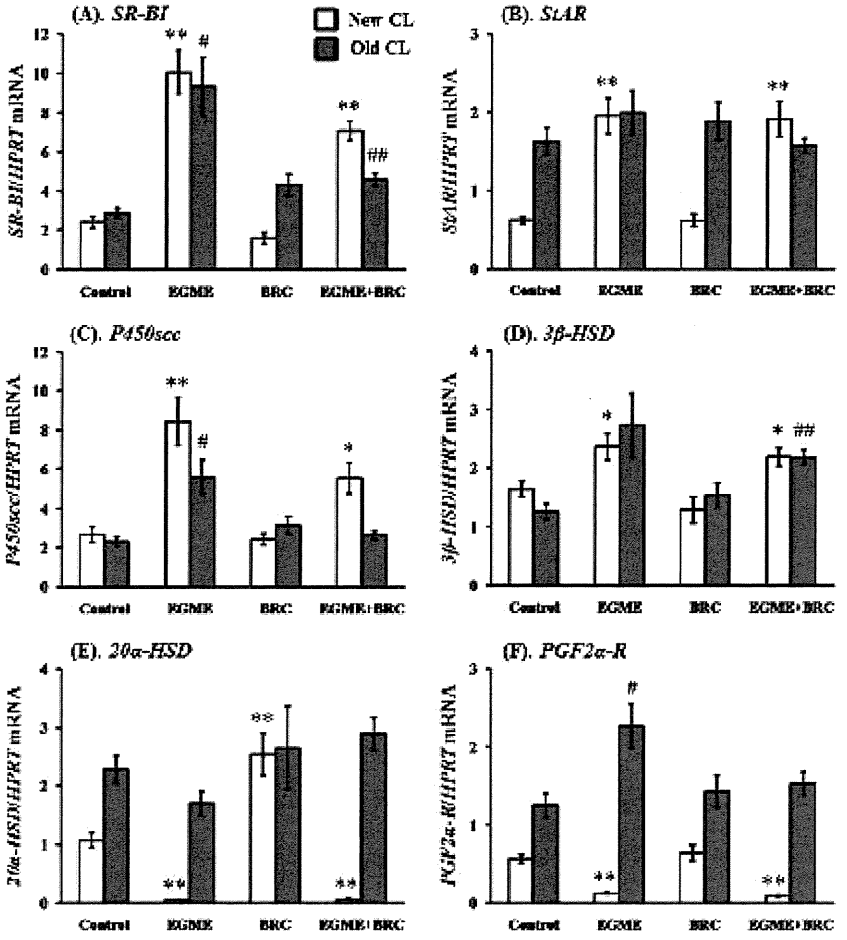

FIG. 7. Effects of EGME, BRC, or EGME and BRC on gene expression levels of steroidogenic and luteolytic factors in new and old CL. Relative mRNA levels of *SR-BI* (A), *StAR* (B), *P450scc* (C), *3β-HSD* (D), *20α-HSD* (E), and *PGF2α-R* (F) are presented. Animals were euthanized 4 h after the last dose ($n = 4$–5). Data are normalized for *HPRT* mRNA levels in each sample and presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, ##$p < 0.01$, #$p < 0.05$).

histopathological changes in the HE-stained specimens following any of the treatments in comparison to the control (data not shown). New CL in the EGME and EGME + BRC groups showed higher intensities of SR-BI, StAR, and P450scc; intensities were significantly higher for StAR in both groups (up to 5.1-fold), SR-BI in the EGME group (1.3-fold), and P450scc in the EGME + BRC group (1.4-fold) in comparison to the controls (Figs. 9A–C). SR-BI was significantly higher in old CL of the EGME + BRC group (1.4-fold) (Fig. 9A). In the BRC group, significantly lower staining intensities were observed in new CL for StAR and 3β-HSD and old CL for 3β-HSD (Figs. 9B and 9D).

DISCUSSION

The focus of this study was to clarify the toxicological pathways by which three ovarian toxicants, EGME, sulpiride,

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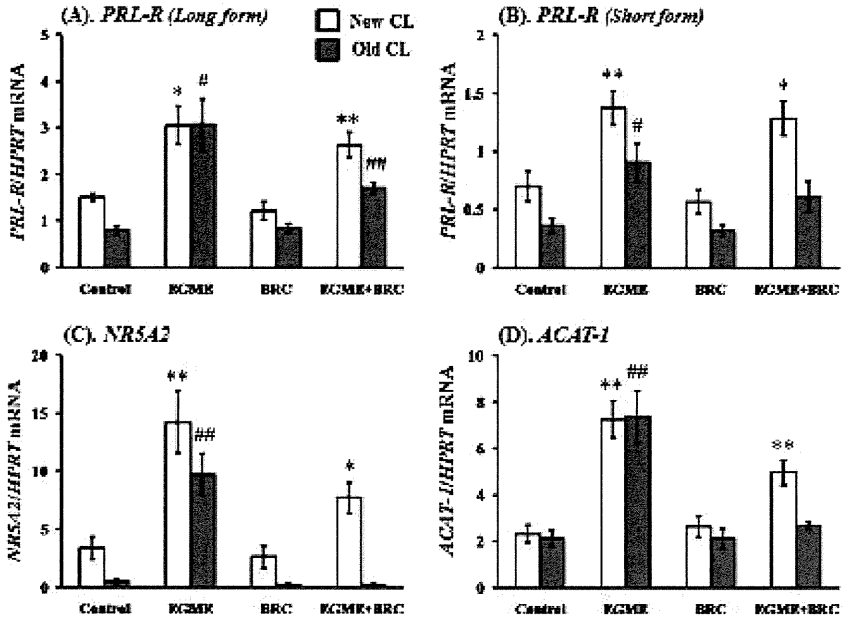

FIG. 8. Effects of EGME, BRC, or EGME and BRC on gene expression levels of *PRL-R (Long form)* (A), *PRL-R (Short form)* (B), *NR5A2* (C), and *ACAT-1* (D) in new and old CL. Animals were euthanized 4 h after the last dose ($n = 4$–5). Data were normalized for *HPRT* mRNA levels in each sample and presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, ##$p < 0.01$, #$p < 0.05$).

and atrazine, affected luteal steroidogenesis in new and old CL *in vivo*. In the present study, all compounds significantly increased the serum P4 level secondary to upregulated expression levels of all steroidogenic factors and downregulation of the *20α-HSD* gene in new CL. The serum E_2, LH, and FSH levels were not altered. Two weeks of treatment with EGME, sulpiride, or atrazine induced similar histopathological changes in luteal cells to what has been previously reported (Davis *et al.*, 1997; Dodo *et al.*, 2009; Shibayama *et al.*, 2009; Yuan and Foley, 2002). These histopathological changes reflect the activation of luteal cells and are associated with increased P4 production activity (Davis *et al.*, 1997; Fraites *et al.*, 2009; Ishii *et al.*, 2009). The present short-term study provides new evidence that new CL are the main targets of these compounds.

EGME as well as sulpiride, a compound which stimulates PRL secretion, increased the serum PRL level in the present study. This is in contrast to a previous study showing suppression of PRL secretion following EGME treatment of female rats (Davis *et al.*, 1997). Although the reason of this discrepancy is unclear, our results represent new evidence that EGME, via the HPG axis, indirectly stimulates luteal pathways in the ovary. This conclusion is further strengthened by the results following coadministration of BRC with EGME, which will be discussed subsequently.

The results of experiment 2 indicate that the gene expression changes of steroidogenic factors correspond to the changes in the immunohistochemical intensities of the factors in new and old CL. The increases in *SR-BI*, *StAR*, *P450scc*, and *3β-HSD* mRNA levels and immunohistochemical intensity levels in new CL following four daily treatments with any of the three compounds represent stimulation of steroidogenesis with resulting P4 production in new CL. Additionally, the increase in *3β-HSD* expression both at the gene transcription and protein expression levels in old CL following treatment with EGME indicates that EGME stimulates steroidogenesis in old CL. Although increases in steroidogenic gene expression were not produced by treatment with the other compounds, a trend to increased immunoreactivity was seen, suggesting that sulpiride and atrazine may also stimulate steroidogenesis in old CL.

The decreases of the luteolytic factor *20α-HSD* mRNA in the new CL from all treatment groups and *PGF2α-R* mRNA in the new CL from the EGME group clearly demonstrate the inhibition of catabolism of P4 into 20α-DHP, indicating a higher P4 production ability in new CL. These results demonstrate that not only promotion of steroidogenesis but also suppression of P4 catabolism in new CL is directly or indirectly induced by EGME, sulpiride, or atrazine treatment.

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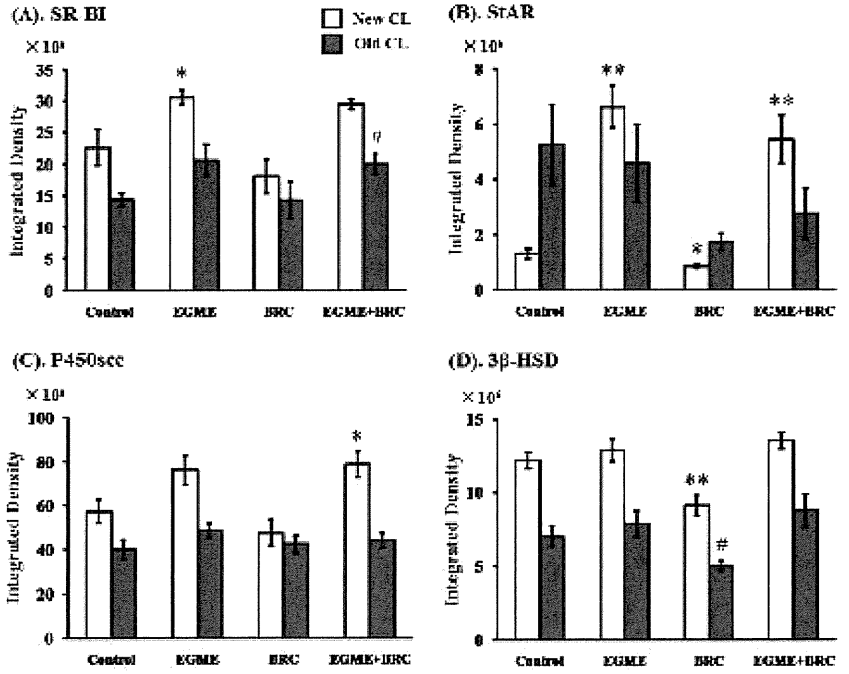

FIG. 9. Effects of EGME, BRC, or EGME and BRC on the immunoreactivities of steroidogenic factors in new and old CL. Integrated densities of SR-BI (A), StAR (B), P450scc (C), and 3β-HSD (D) are presented. Animals were euthanized 4 h after the last dose ($n = 4$–5). Data are presented as the mean ± SEM, with asterisks and hashes indicating significant differences as compared with the controls (**$p < 0.01$, *$p < 0.05$, #$p < 0.05$).

Consistent with the role of PRL as an upregulator of steroidogenic factors and a downregulator of 20α-HSD in the rat ovary (Stocco *et al.*, 2007), the present study clearly showed that the stimulation of P4 secretion by sulpiride was caused by increased PRL secretion. The concurrent administration of EGME with BRC, a D2 agonist, in experiment 3 demonstrated that EGME stimulated P4 secretion, upregulated steroidogenic factor expression levels, and downregulated luteolytic factor expression levels in new CL without PRL hypersecretion. These findings taken in the context of consistent observations *in vitro* (Almekinder *et al.*, 1997; Davis *et al.*, 1997) suggest that EGME has a direct luteal stimulatory effect *in vivo*. Although the immunohistochemical intensities of P450scc and 3β-HSD in new and old CL of EGME group were significantly higher than controls in experiment 2, there was no significant change between them in experiment 3. These inconsistencies were attributed to differences in the conditions for tissue fixation; ovaries were fixed in 4% paraformaldehyde and 10% vol neutral-buffered formalin in experiments 2 and 3, respectively.

In other steroidogenesis-related genes, increases in both forms of *PRL-R* in new CL following EGME or sulpiride treatment may reflect increased PRL secretion because they have been upregulated in pregnant female rats (Dunaif *et al.*, 1982; Telleria *et al.*, 1997). Additionally, EGME and sulpiride remarkably increased *NR5A2* mRNA level in new CL. *NR5A2* is a crucial factor in PRL-regulated P4 secretion (Falender *et al.*, 2003; Labelle-Dumais *et al.*, 2007; Lin *et al.*, 2003; Saxena *et al.*, 2007). Therefore, the increased expression of the *NR5A2* gene might be related to the PRL-stimulating effect of EGME and sulpiride. Because the *SF-1* gene, which belongs to the same nuclear receptor family as *NR5A2*, regulates the E_2 and P4 synthesis in granulosa cells (Falender *et al.*, 2003; Saxena *et al.*, 2007), its expression in CL did not change in any treatment groups. Upregulation of the *ACAT-1* gene, which contributes to the storage of free cholesterol in lipid droplets in luteal cells (Stouffer, 2006), was also observed in new and old CL following the EGME or sulpiride treatment. This change seems to contribute the histopathological luteal hypertrophy by repeated administration of these compounds. Intriguingly, coadministration of EGME and BRC produced similar changes in gene expression levels of *PRL-R*, *NR5A2*, and *ACAT-1* in new CL. These observations allude to the possibility that the direct luteal stimulation mechanism of EGME is similar to PRL

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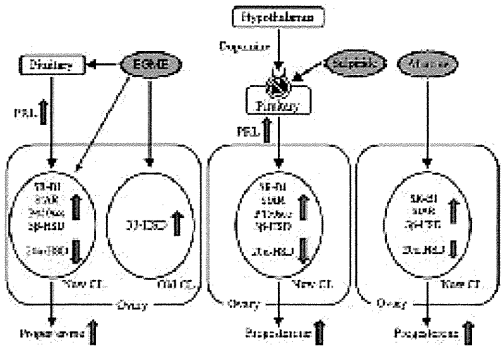

FIG. 10. Overview of the luteal effects of EGME, sulpiride, and atrazine. EGME upregulates steroidogenic factor expressions and downregulates luteolytic factor expression. This results in an increase in P4 secretion from new CL by both direct and indirect (PRL dependent) pathways. EGME also directly stimulates the expression of 3β-HSD in old CL. Sulpiride stimulates PRL secretion sustainably by suppressing the dopaminergic activity in the anterior pituitary. This results in the activation of new CL and increased P4 secretion. Atrazine also stimulates P4 secretion in new CL by upregulating SR-BI, StAR, and 3β-HSD expression levels and downregulating 20α-HSD expression level. Gray arrows represent the increases or decreases of the luteal gene/protein expression levels or hormone secretion.

dependent stimulation pattern. Thus, two pathways likely mediate the luteal stimulatory effect of EGME on the ovary and consist of a direct pathway and an indirect pathway, which depends on PRL hypersecretion.

Previous reports have described a neuroendocrine effect of atrazine in rats. Atrazine inhibits the preovulatory surge of LH and PRL by disrupting the hypothalamic control (Cooper *et al.*, 2000; Foradori *et al.*, 2009). Although these reports explain the disruption of estrous cyclicity by atrazine treatment, the cause of the increased P4 level cannot be explained by these observations. Fraites *et al.* (2009) and Laws *et al.* (2009), however, recently reported that atrazine or its chlorinated metabolite, desisopropylatrazine, increased circulating P4 levels by activating the hypothalamic-pituitary-adrenal axis in male and female rats. The present results demonstrated no increase in serum PRL level following atrazine treatment. In addition, no morphological change was observed in the adrenals in the present study, suggesting a possibility of a direct stimulatory effect of atrazine on CL. Because the ovary is the main site of P4 secretion in female rats, P4 production from the adrenal likely does not represent a significant target of atrazine treatment in female.

With regard to the other steroidogenesis-related genes (*PRL-R*, *NR5A2*, and *ACAT-1*), the lack of change in expression levels following atrazine treatment suggests that its mechanism differs from that of EGME or sulpiride. Atrazine has been shown to increase the concentration of cyclic adenosine monophosphate (cAMP) (Sanderson *et al.*, 2002), inhibit phosphodiesterase (Roberge *et al.*, 2006), induce *P450scc* gene expression, and activate phosphatidylinositol 3-kinase (PI3K) signaling cascades (Suzawa and Ingraham, 2008) *in vitro*. The present study did not confirm any of these findings and suggests that further experiments are needed to clarify the mechanism of atrazine.

An overview of the possible pathways mediating the effects of EGME, sulpiride, or atrazine on luteal cells is summarized in Figure 10. We propose that EGME stimulates PRL secretion from the pituitary with resulting upregulation of steroidogenic factors and downregulation of luteolytic factor expression levels. This results in increased P4 secretion in new CL. In addition, EGME directly stimulates new CL in a manner similar to PRL and also directly stimulates steroidogenesis in old CL. The luteal stimulation effect of sulpiride is solely the result of disruption of the HPG axis producing hypersecretion of PRL from pituitary (indirect effect). Our results indicate that atrazine directly stimulates P4 secretion in new CL in a PRL independent manner. In conclusion, the present study provides new insights regarding the differential pathways mediating the stimulation of P4 secretion by the ovarian toxicants EGME, sulpiride, and atrazine in female rats *in vivo*. Additional studies are needed to confirm the direct effect of EGME and atrazine on luteal cells and to elucidate its mechanism.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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