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Source: Zoological Science, 15(4) : 525-529

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/0289-0003(1998)15[525:EOOAPO]2.0.CO;2

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ZOOLOGICAL SCIENCE **15**: 525–529 (1998)
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Effects of Ovariectomy and Prolactin on Mammary Gland Expressions of Transforming Growth Factor α and Epidermal Growth Factor Receptor mRNAs in Mice

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ABSTRACT—Beginning 15 days after ovariectomy (OVX), a high mammary tumor strain of SHN virgin mice at 3 months of age received subcutaneous injections of danazol (0.5 μg / 0.1 ml olive oil, once a day), perphenazine (0.05 mg / 0.1 ml saline, twice a day) or ovine prolactin (oPRL: 0.25 mg / 0.05 ml buffer, twice a day) for 3 days to modulate their circulating PRL levels. The serum PRL level was significantly decreased by danazol and increased by perphenazine compared to the intact and OVX-control groups. The expression of both transforming growth factor α (TGFα) mRNA and epidermal growth factor receptor (EGFR) mRNA in the mammary gland was increased by danazol. However, TGFα mRNA expression was decreased by perphenazine. Meanwhile, mammary end-bud formation was inhibited in danazol-treated group. All findings suggest that the manifestation of the effect of TGFα on mammary gland is rather suppressed by PRL, while mammary gland growth needs the participation of PRL; in other words, PRL is dominant to TGFα on the mammary gland growth. OVX resulted in a significant decrease of TGFα mRNA expression in the mammary gland despite of little alteration in serum PRL, confirming the previous observations. The similar trend was observed in ICR mice; however, the response to hormonal modulation is generally less susceptible than SHN mice.

INTRODUCTION

Both estrogen and prolactin (PRL) are key hormones for the normal, preneoplastic and neoplastic growth of mammary glands (Nagasawa *et al*., 1986), and these hormones have been proposed to stimulate cell growth indirectly through an autocrine or paracrine mechanism by enhancing the production of peptide factors, as well as by direct effects. Transforming growth factor α (TGFα) participates in the growth of normal and neoplastic mammary glands (Perroteau *et al*., 1986; Derynck *et al*., 1987; Liu *et al*., 1987; Zajchowski *et al*., 1988; Valverius *et al*., 1989; Matsui *et al*., 1990; Halter *et al*., 1992; Mizuno *et al*., 1994), acting through binding to epidermal growth factor receptor (EGFR) (Todaro *et al*., 1980). However, the information of the participation of estrogen and/or PRL on this process is rather sporadically. TGFα is suggested to be modulated by estrogen (Dickson *et al*., 1986; Arteaga *et al*., 1988; Bates *et al*., 1988; Manni *et al*., 1991). Prusheik *et al*. (1997) suggested that PRL inhibited EGFR signaling.

In the present study, the effects of OVX and of the modulation of circulating PRL on expressions of TGFα and EGFR mRNAs in the mammary gland were examined in virgin mice. Perphenazine (Singtripop *et al*., 1991) and danazol (Singtripop *et al*., 1992) were used for the stimulant and the inhibitor of pituitary PRL release, respectively. The effect of ovine prolactin (oPRL) in this process was also examined.

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MATERIALS AND METHODS

Animals and treatments

The mice used were SHN/Mei virgin mice maintained in our laboratory by strict brother x sister mating (Nagasawa *et al*., 1976) and Jcl: ICR virgin mice purchased from CLEA Japan (Tokyo, Japan). At 3 months of age, mice of each strain were divided into five groups. Four groups were bilaterally ovariectomized via a dorsal approach under anesthesia by pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, USA) and the remaining one group underwent a sham operation and served as the intact control. Beginning 15 days after the operation, the OVX group received subcutaneous injections of saline (Otsuka Seiyaku, Tokyo, Japan; 0.1 ml, twice a day) or olive oil (Kozakai Seiyaku, Tokyo, Japan; 0.1 ml, once a day), the data of which were pooled in the Results section, since they differed little (OVX control); danazol (Sigma Chem. Co., St. Louis, MO, USA; 0.5 μg / 0.1 ml olive oil, once a day), perphenazine (Sigma; 0.05 mg / 0.1 ml saline, twice a day) or ovine PRL (AFP-10677C, NIDDK; 0.25 mg / 0.05 ml buffer, twice a day) for 3 days and on the morning of day 4. The mice undergone the sham operation were treated as was in the OVX control. All mice were killed one hour after

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Table 1. Sequence of primers used in RT-PCR and size of predicted products amplified

Primer		Primer location	Sequence	Size (bp) of predicted product
TGFα	sence	21–41	5'-GGACAGCTCGCTCTGCTAGCG-3'	347
	antisense	267–350	5'-TGGATCAGCACACAGGTG-3'	
EGFR	sense	234–253	5'-GGAGGAAAAGAAAGTCTGCC-3'	304
	antisense	537–518	5'-CCCATAGTTGGACAGGATGG-3'	

the last injection under light ether anesthesia and blood was collected from the trunk.

All procedures were carried out according to the USA NIH Guide for the Care and Use of Laboratory Animals.

Throughout the experiment, mice were kept in plastic cages (18 × 30 × 13 cm) with wood shavings, maintained in an animal room, which was air-conditioned (20-22°C and 60-70% relative humidity), artificially illuminated (14 hr of light from 5:00 AM to 7:00 PM) and ventilated (16 times / hr) and provided with commercial pellets (Lab MR-Breeder; Nihon Nosan Kogyo, Yokohama, Japan) and tap water *ad libitum*.

Serum PRL level

Blood collected was left at room temperature for 6 hr, kept in the refrigerator overnight and centrifuged at 1,000 × g for 20 min at 4°C. Serum was stored at –20°C. The PRL level was determined by a homologous radioimmunoassay.

End-bud formation in the mammary glands

At autopsy, unilateral third thoracic mammary glands were prepared for the wholemount evaluation and were examined under 10-fold magnification. The degree of end-bud formation was rated from 1 to 7 in increments of 1 (Nagasawa *et al.*, 1980).

Northern blot analysis

At autopsy, bilateral inguinal and unilateral third thoracic mammary glands were immediately removed, pooled and stored at –70°C. RNA was extracted from 200-250 mg of frozen tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The RNA concentration was determined at 260 nm by spectrophotometer. Each 20 μg of RNA from each sample was applied to the nylon membrane (Immobilon™; Millipore, Bedford, MA, USA) with a Bio-dot manifold (Bio-rad, Hercules, CA, USA) and then the RNA was fixed to membrane using UV crosslinking. The transforming growth factor α (TGFα) and epidermal growth factor receptor (EGFR) complementary DNA (cDNA) fluolescein-labelled probes were labelled using the Gene Images random prime labelling module kit (Amersham, Buckinghamshire, England). Northern blot analysis was performed by hybridization with this fluolescein-labelled cDNA probes for 16 hr at 65°C and was tested by using the Gene Images CDP-Star detection module kit (Amersham). The expression intensities of TGFα mRNA and EGFR mRNA were analyzed using Multi-Analyst (Bio-rad).

The mouse TGFα and EGFR cDNA probes used were synthesized from the mammary gland of the mouse by reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR reaction for the probes of both TGFα and EGFR was performed for 40 cycles (1 cycle=94°C for 1 min, 55°C for 1 min, 72°C for 2 min) in a program temperature control system (Astec, Fukuoka, Japan). The sequence of the primers used for RT-PCR are shown in Table 1 (Avivi *et al.*, 1991; Snedeker *et al.*, 1991; Vaughan *et al.*, 1992). The other conditions were the same as detailed previously (Harigaya *et al.*, 1994; Tsunoda *et al.*, 1997).

In each assay, samples from all 5 groups were determined simultaneously and the values were expressed in terms of the percentages against that of the intact control.

Statistics

The statistical significance of differences in each parameter among groups were evaluated by Duncan's multiple range test.

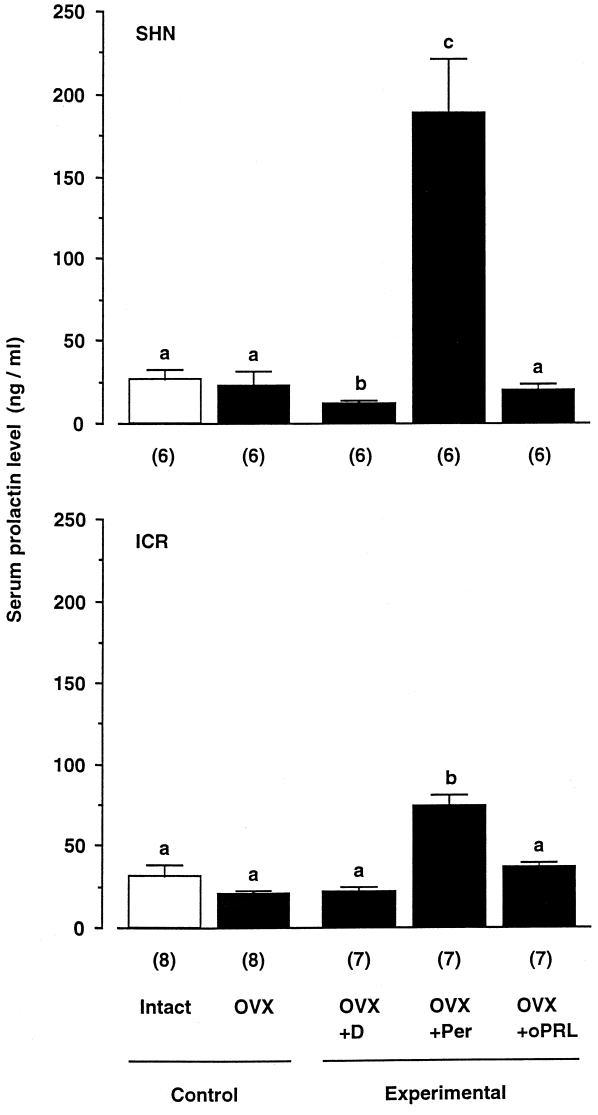

Fig. 1. Serum prolactin level in each group (mean ± SEM). Numbers of estimates are in parentheses. OVX, ovariectomy; D, danazol; Per, perphenazine; oPRL, ovine prolactin. [a–c] Values with different superscripts differ significantly at $P < 0.05$ or 0.01.

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RESULTS

Serum PRL level (Fig. 1)

The serum PRL level was significantly higher in the perphenazine-treated group than in the intact and OVX-control groups in both SHN and ICR mice. Moreover, in SHN mice, the serum PRL level of the danazol-treated group was significantly lower than those of the intact and OVX-control groups. The level of the danazol-treated group of ICR was also lower than that of the intact-control group, but the difference was not significant. Little difference was seen among the other groups in the serum PRL level in both SHN and ICR mice.

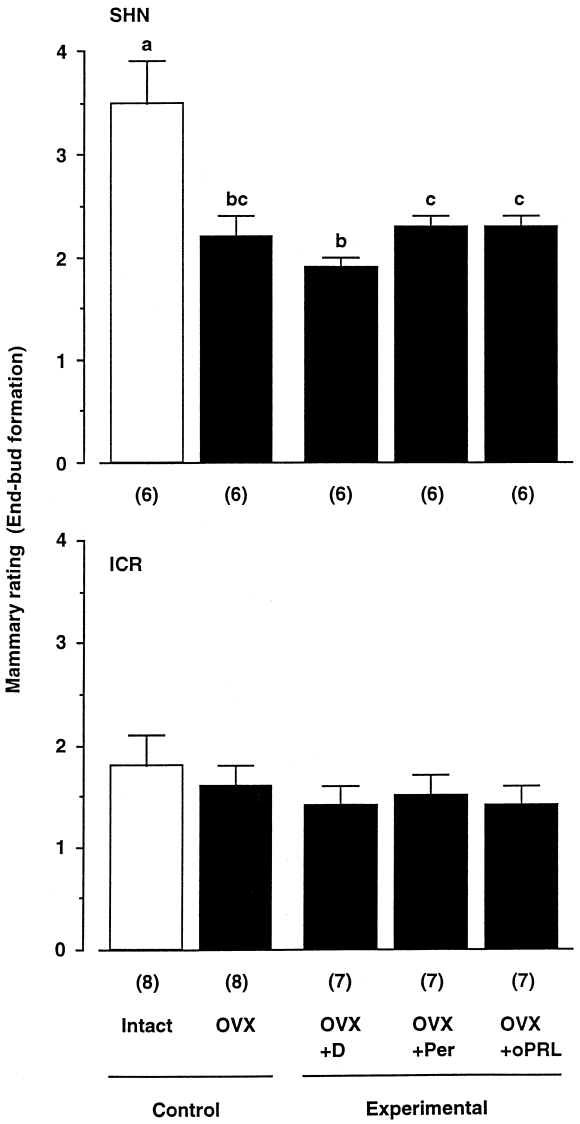

Fig. 2. Mammary rating as an index of end-bud formation in each group (mean ± SEM). Numbers of estimates are in parentheses. OVX, ovariectomy; D, danazol; Per, perphenazine; oPRL, ovine prolactin. [a–c] Values with different superscripts differ significantly at $P < 0.05$ or 0.01.

End-bud formation in mammary gland (Fig. 2)

In SHN mice, mammary rating as an index of end-bud formation was decreased significantly by OVX itself compared to the intact control group. The rating was further lower in the danazol-treated group than in the others.

Little difference in the rating was seen among groups of ICR mice.

Expression of TGFα mRNA and EGFR mRNA in the mammary gland

In the first experiment, the northern blot analysis with agalose gel electrophoresis was performed to detect the mRNA size, but the band could not be detected. Then, the dot-blot analysis was carried out to determine the quantities of the mRNA.

The results of transforming growth factor α (TGFα) mRNA are shown in Fig. 3. In SHN mice, TGFα mRNA expression was significantly lower in the OVX-control group and perphenazine-treated group than in the intact control group. TGFα mRNA expression of the ovine prolactin-treated group was also lower than that of the intact control group, while the difference was not significant. In ICR mice, the perphenazine-treated group and oPRL-treated group tended to be lower than the intact control group in the TGFα mRNA expression.

As presented in Fig. 4, epidermal growth factor receptor (EGFR) mRNA expression was apparently higher in the danazol-treated group than in the intact control group in both SHN and ICR, while the difference in SHN was not significant owing to the large variation.

DISCUSSION

The present results show that a decrease of the circulating PRL by danazol induced an apparent elevation of both transforming growth factor α (TGFα) mRNA and epidermal growth factor receptor (EGFR) mRNA in the mammary glands of SHN mice. Furthermore, TGFα mRNA expression in the mammary gland was decreased by perphenazine associated with an increase in the PRL level. These findings indicate that PRL acts suppressively on the manifestation of effects of TGFα on the mammary glands. On the other hand, mammary end-bud formation was lower in the danazol group than in the group treated with perphenazine or oPRL. The findings would indicate that the stimulating effects of TGFα on the mammary glands would need the simultaneous participation of PRL; in other words, the role of PRL on mammary gland growth is dominant to that of TGFα.

TGFα expression in the mammary gland was decreased by OVX compared to the intact control group despite of little difference in serum PRL level. This confirmed the previous reports that TGFα is modulated by estrogen (Dickson *et al.*, 1986; Arteaga *et al.*, 1988; Bates *et al.*, 1988; Manni *et al.*, 1991).

The differences among groups in the parameters examined except EGFR mRNA were smaller in ICR than in SHN. This would reflect that mammary gland of ICR is less suscep-

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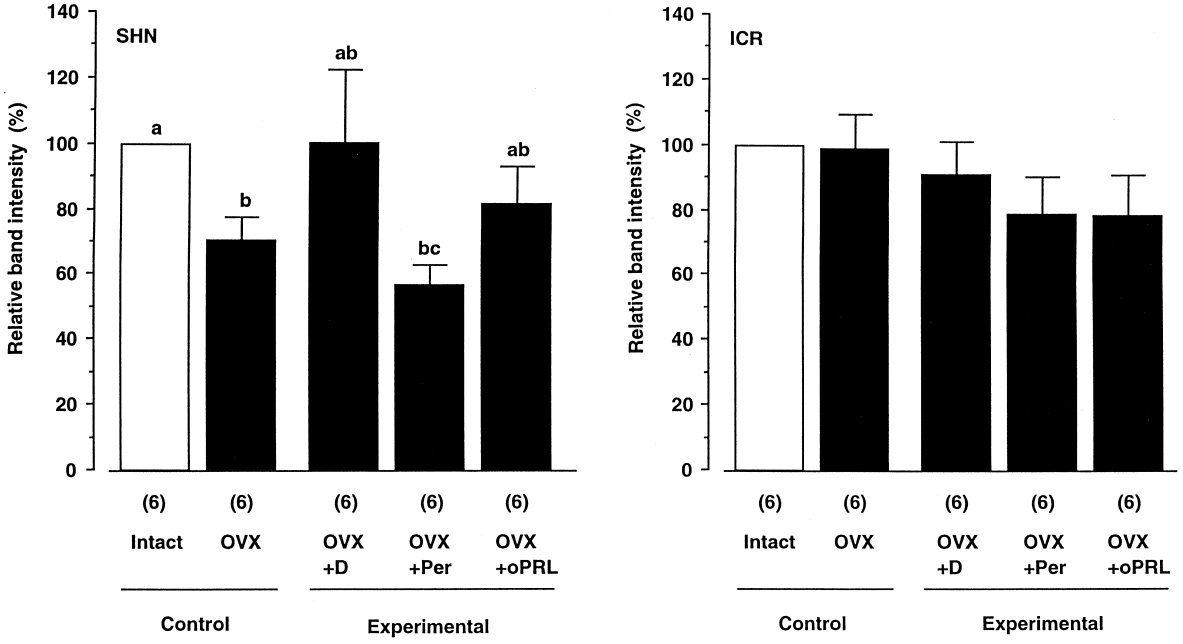

Fig. 3. Northern blot analysis of TGFα mRNA expression in the mammary glands in each group. Relative band intensity, which is expressed as a percentage against the intact control (mean ± SEM). Numbers of estimates are in parentheses. OVX, ovariectomy; D, danazol; Per, perphenazine; oPRL, ovine prolactin. [a–c] Values with different superscripts differ significantly at $P < 0.05$ or 0.01.

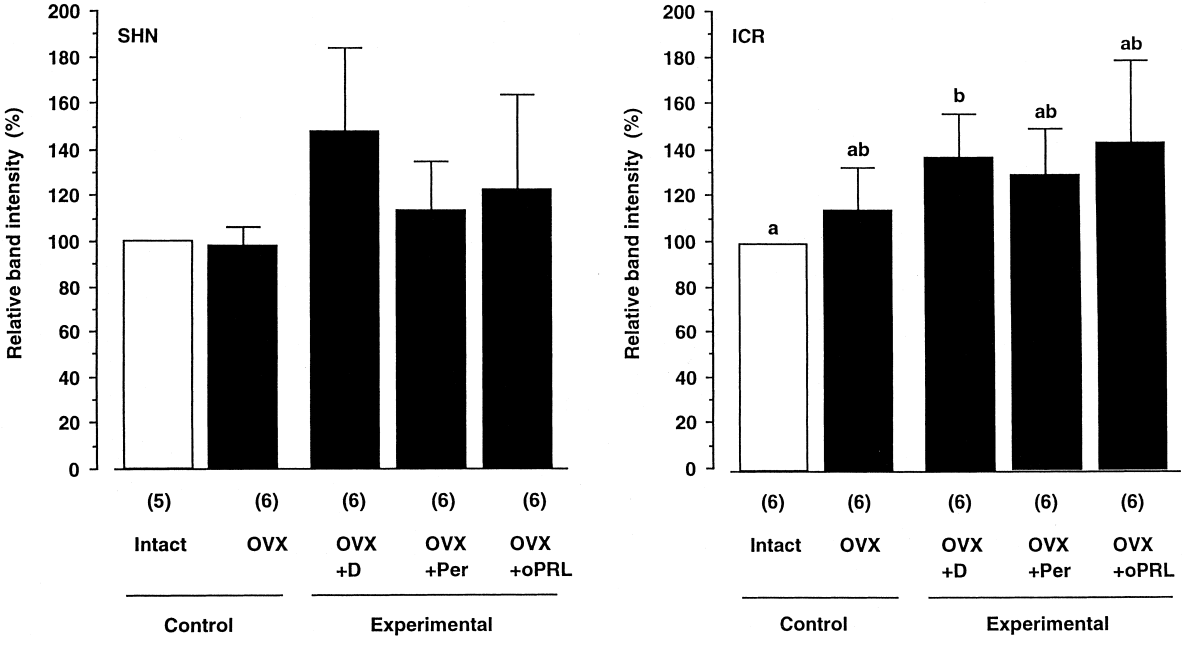

Fig. 4. Northern blot analysis of EGFR mRNA expression in the mammary glands in each group. Relative band intensity, which is expressed as a percentage against the intact control (mean ± SEM). Numbers of estimates are in parentheses. OVX, ovariectomy; D, danazol; Per, perphenazine; oPRL, ovine prolactin. [a–b] Values with different superscripts differ significantly at $P < 0.05$.

tible to OVX and the modulation of circulating PRL than that of SHN. Incidentally, in mice, high mammary gland susceptibility to mammotropic hormones is a characteristic of high mammary tumor strains (Nagasawa and Yanai, 1978).

ACKNOWLEDGMENTS

We thank Dr. T. Harigaya, Professor of Functional Anatomy of this University for his invaluable advice and continued interest, Professor A. F. Parlow, Pituitary Hormones & Antisera Center, Harbor UCLA-Medical Center, Torrance, CA, for the radioimmunoassay kit for mouse PRL and Professor K. Wakabayashi, Gunma University, for goat anti-rabbit IgG serum. The ovine PRL (AFP-10677C) was

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donated by the National Hormone and Pituitary Program, NIDDK, Bethesda, MD, USA, which is also acknowledged.

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(Received March 16, 1998 / Accepted April 14, 1998)

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