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Estradiol-17β Silastic Implants in Female *Rana ridibunda* Depress Thyroid Hormone Concentrations in Plasma and the *in Vitro* 5′-Monodeiodination Activity of Kidney Homogenates

G. VANDORPE AND E. R. KÜHN

Laboratory of Comparative Endocrinology, Zoological Institute, Catholic University of Leuven, Naamsestraat 61, B-3000 Leuven, Belgium

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Estradiol-17β-containing silastic tubings were implanted in female *Rana ridibunda*. Preliminary data, concerning *in vitro* incubations of such tubings in saline media, revealed that high concentrations of estradiol were released out of the tubings in the incubation medium. Compared to control-implanted frogs, the frogs that had the estradiol tubings implanted for 30 days showed a significant increase of the plasma estradiol concentration, the ovarian estradiol concentration, and the weight of the oviduct. Plasma triiodothyronine (T_3) levels, plasma thyroxine levels, and the *in vitro* T_3 production in the kidney homogenates were significantly decreased. These results indicate that high estradiol levels not only influence the gonadal axis, but also cause important effects on the thyroidal axis. © 1989 Academic Press, Inc.

Several authors have suggested interrelationships between reproductive status and thyroid status in vertebrate species. Effects of estrogens on thyroidal axis are described in fishes (Olivereau *et al.*, 1981; Milne and Leatherland, 1980; Leatherland, 1985; Chakraborti *et al.*, 1984; MacLatchy *et al.*, 1986; Mackenzie, 1986; Cyr *et al.*, 1988) birds, (different authors, review Sharp and Klandorf, 1985; Hoshino *et al.*, 1987), and mammals (Galton, 1971; Fisher and d'Angelo, 1971; De Lean *et al.*, 1977; Sawhney *et al.*, 1978a,b; Glinoer *et al.*, 1977). In the class of the amphibians, however, little or no information is available about the direct effects of estradiol-17β (E_2) on the thyroidal axis. Studies performed on the seasonal cycle of *Dicroglossus occipitalis* (Kühn *et al.*, 1987) suggest an inverse relationship between plasma concentrations of thyroxine (T_4) and E_2. We therefore have examined the effects of E_2 on the thyroid axis in female *Rana ridibunda*.

MATERIALS AND METHODS

In vitro incubations of estradiol-17β silastic tubings. In a preliminary experiment, three pieces of silastic tubing (Silastic, Medical Grade Tubing, Dow Corning, inside diameter 1.47 mm, outside diameter 1.96 mm), of 7 cm length, were filled with dry estradiol-17β powder (Sigma; tubing 1: 42.7 mg E_2, tubing 2: 40.3 mg E_2, tubing 3: 33.5 mg E_2), sealed at both ends, and incubated in a saline medium (NaCl; 0.65%). The E_2-containing tubings were incubated separately in plastic containers filled with 45 ml saline medium, which were shaken continuously (40 times/min). At regular time intervals the medium was renewed (tubing 1: 12 and 24 hr, tubing 2: 2 hr, tubing 3: 24 hr). Estradiol was assayed in the successive media by a radioimmunoassay (RIA), using a commercial kit (Biotecx, USA).

Implantation of estradiol tubings in living frogs. The experiment was performed on female *R. ridibunda*, purchased from the "Centre d'élevage d'animaux de laboratoire d'Ardenay (Le Breil sur Mérize, France). Pieces of silastic tubing (length: 7 cm) were filled with dry estradiol-17β powder (Sigma; E_2 weight/tubing: mean, 30.45 mg; minimum, 28.2 mg; maximum, 33.1 mg) and implanted under the dorsal skin in anesthetized frogs. The frogs were anesthetized in a 0.16% MS 222 solution. Empty tubings were implanted in the same way in frogs which served as the control group. Thirty days after the tubings were implanted, the frogs were killed by pithing (implantation data: February 17; day of pithing: March 18). A blood sample was taken directly from the ventricle and the organs were excised.

Analysis of the samples. The plasma samples were extracted with acetone (according to Darras and

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Kühn, 1982) prior to RIA of triiodothyronine (T_3) and T_4. Hormone concentrations were assayed by using tracer obtained from Amersham International (UK), rabbit T_3 antiserum from Mallinckrodt (GFR), and a laboratory-raised rabbit T_4 antiserum (final titer 1/8250). T_3 and T_4 antiserum did not show any cross-reactivity for T_4 concentrations until 5 ng/ml and T_3 concentrations up to 2 ng/ml frog plasma. The T_3/T_4 ratio is expressed as $T_3/T_4 \times 100$.

The kidneys were homogenized in ice-cold sodium phosphate buffer (0.15 *M*, pH 7.4, 0.5 g kidney/3.2 ml) with an Ultra-Turrax homogenizer. The 5'-monodeiodination (5'-D) activity was measured in the kidney homogenates according to Decuypere *et al.* (1983) using ice-cold polyoxyethylene monolauryl ether (Brij 35, 1.25%, Serva) instead of ethanol to stop the reaction. In this method, T_4 (8 μg/g kidney) was added as a substrate, reducing conditions were obtained by adding 1,4-dithio-DL-threitol (DTT), and EDTA (8 and 6 m*M*, respectively, in a volume of 100 μl) was also added to the homogenates. The homogenates together with the reagentia were incubated at 37° for 1 hr. T_3 was measured with a RIA, analogous to the RIA used for the plasma samples; the parallelism between dilutions of homogenates and the standard curve was tested before. The given T_3 values are the result of the values measured in the homogenates, deducted with corresponding blank values. A blank value is obtained by adding T_4 to a tissue homogenate at the end of the incubation, to take into account the cross-reactivity of T_4 in the T_3 RIA and the presence of T_3 in the homogenates before adding T_4.

E_2 in the plasma was measured by RIA following extraction with a cyclohexane–ethylacetate mixture (v/v) using a commercial kit (Biodata, Italy). E_2 in the ovaries was measured by the following method. From each frog a piece of ovarian tissue of 0.5 g weight was homogenized in methanol 70% with an Ultra-Turrax homogenizer. After an incubation period of 24 hr at 4°, the homogenates were centrifugated and the supernatant was evaporated under nitrogen. Subsequently the dried samples were extracted with a diethylether/water mixture (8/1, v/v). The ether layer was then removed and dried under nitrogen. E_2 was measured by a RIA with the same commercial kit after the samples have been redissolved in a sodium–phosphate buffer. The weight of the oviduct was measured and expressed as the percentage of the weight of the ovary.

Statistical analysis of data was made by one-way analysis of variance (ANOVA). Correlation coefficients between the data were calculated.

RESULTS

In Vitro Incubations of Estradiol-17β Silastic Tubings

The release of E_2 into a saline environment by three different tubings was tested. Following an initial release of more than 3000 ng/hr, values between 400 to 1000 ng/hr were obtained after 1 day. After 19 days of incubation a release of 500 ng/hr was still present.

Implantation of Estradiol Tubings in Living Frogs

Figure 1 summarizes the E_2 concentration in the plasma and the ovaries as well as the oviduct weight of both control and E_2-tubing frogs. After a period of 30 days, during which E_2-tubings were implanted, very significant increases in both plasma and ovarian E_2 concentration are observed (E_2 plasma: $P < 0.001$; E_2 ovary: $P < 0.001$). The weight of the oviduct is also significantly elevated in the E_2-tubing frogs compared to the control frogs ($P < 0.05$). A positive correlation exists between E_2 in the ovaries and the plasma ($P < 0.01$).

Figure 2 summarizes the plasma T_3 and T_4 concentration and the plasma T_3/T_4 ratio in the plasma samples collected 30 days after the tubings were implanted. T_3 values, as well as T_4, are decreased significantly (T_3: $P < 0.01$; T_4: $P < 0.05$). The T_3 and T_4 values of the E_2-tubing frogs are respectively about five and two times lower compared to the values of the control frogs. A nearly significant decrease of the T_3/T_4 ra-

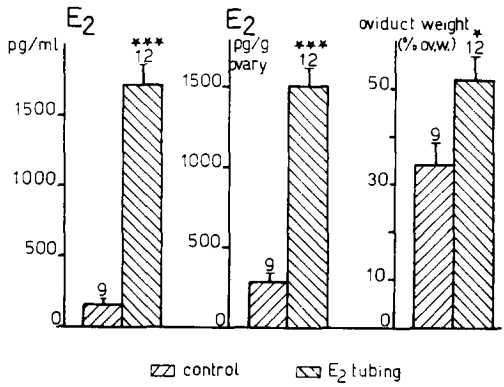

FIG. 1. Plasma E_2 concentration (pg/ml), ovarian E_2 concentration (pg/g ovary), and oviduct weight (percentage of the ovary weight) of female *R. ridibunda* with implanted E_2 tubings and in control frogs. Mean ± SEM; number of animals as indicated.

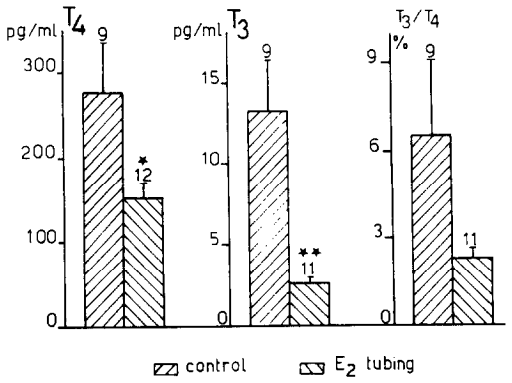

FIG. 2. Plasma T_4 (pg/ml), plasma T_3 (pg/ml), and the plasma T_3/T_4 ratio (percentage) in female *R. ridibunda* with implanted E_2 tubings and in control frogs. Mean ± SEM; number of animals as indicated.

tio is found in the E_2-tubing group ($P = 0.055$) compared to the control group.

Figure 3 summarizes the *in vitro* T_3 production in the kidney homogenates. This T_3 production is about two times lower in the E_2-tubing frogs compared to the control frogs ($P < 0.05$). The plasma T_3/T_4 ratio is positively correlated with the *in vitro* 5′-D activity in the kidney homogenates ($P < 0.05$).

DISCUSSION

The present study clearly indicates that

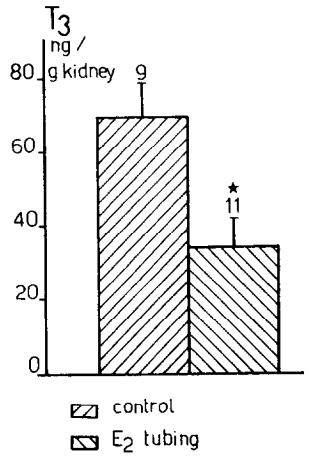

FIG. 3. T_3 production (ng/g kidney) in kidney homogenates incubated for 1 hr at 37° in the presence of 8μg T_4/g kidney and 100 μl DTT + EDTA (8 and 6 m*M*, respectively). Mean ± SEM; number of animals as indicated.

administration of high E_2 concentrations not only causes changes in several gonadal parameters, but also has remarkable effects on the thyroidal axis.

The release of E_2 from the silastic tubings results in high levels of E_2 in plasma and ovary, indicating that estradiol of nonovarian origin can accumulate in the ovary. The important role of the estrogens in the development of the oviduct is illustrated in our experiment, since a higher oviduct weight is found in the E_2-tubing frogs. The strong elevations of plasma E_2 in the E_2-tubing frogs (about 10 times above control animals) could be regarded as nonphysiological. However, this value is only 2.3 times higher than the maximal E_2 concentrations found during reproduction of female *R. ridibunda* (G. Vandorpe *et al.*, unpublished observation).

After E_2 treatment, there is a twofold decrease in plasma T_4 and the 5′-D activity is two times lower. These two factors could contribute to the fivefold decrease of the plasma T_3 concentration. Although conflicting effects of estradiol treatment upon the thyroidal axis have been described in fishes, which are also poikilotherm vertebrates, several authors reported effects similar to the ones found in our study. Estradiol administration in the eel, *Anguilla anguilla,* resulted in a significant decrease of plasma T_3 and T_4 (Olivereau *et al.*, 1981). Leatherland (1985) performed an experiment on the rainbow trout, *Salmo gairdneri.* The trouts were injected intraperitoneally with 1 ml of warm hydrogenated coconut oil containing 30 mg estradiol. These "implants" of hydrogenated coconut oil solidified within the peritoneal cavity within 1–2 min after returning the fishes to cold water and were still evident 14 days later, at which time approximately 50% of the coconut oil had been absorbed. This experimental setup shows some resemblance with our experiment on frogs and indeed similar results were obtained: a significant decrease of plasma T_3 and T_4 was observed, while the T_3/T_4 ratio was unchanged. MacLatchy

et al. (1986) and Cyr *et al.* (1988) reported a decrease of the hepatic T_4 5′-D activity in rainbow trout after estradiol injection. This observation is in agreement with our finding that T_3 production in the kidney homogenates is depressed in the E_2-tubing frogs, taking into account that the kidney, rather than the liver, is an important organ for peripheral monodeiodination activity in frogs (Vandorpe *et al.*, 1987).

In order to explain the effects of estradiol on the thyroidal axis, the following possibilities may be suggested. In *R. ridibunda, R. temporaria,* and *R. esculenta* it has been shown by Jacobs and co-workers (1988) that synthetic luteinizing hormone-releasing hormone is able to cause a significant increase in plasma T_4 which is mediated via the pituitary. These findings indicate that common hypothalamic control exists for the thyroidal and gonadal axis in frogs. Negative feedback actions exerted by the high estradiol levels could result in a decreased thyroid function or thyroidal secretion. On the other hand, Leatherland (1985) also showed that estradiol treatment in rainbow trout resulted in a decrease of the thyrotropic activity of TSH, indicating that the thyroid of estradiol-treated fishes is less responsive to exogenous TSH stimulation. Further experiments on frogs are needed to investigate if this depressed responsiveness of the thyroids to TSH after estradiol treatment, which can be responsible for decreased T_4 and T_3 levels, also occurs in frogs. On the other hand, the role of E_2 in the peripheral 5′-D activity also needs further investigation because the effect of E_2 on this level is not yet known.

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