ESTROGEN AND L-DIHYDROXYPHENYLALANINE INDUCED CHANGES IN HYPOTHALAMIC BIOGENIC AMINE LEVELS AND SERUM LH IN THE EWE 1,2

J. E. Wheaton, Susan K. Martin and F. Stormshak 3

Oregon State University, 4 Corvallis 97331

Summary

Experiments were conducted to determine the ability of exogenous L-dihydroxyphenylalanine (L-dopa) and 17β-estradiol to alter the concentrations of hypothalamic biogenic amines and affect the release of luteinizing hormone (LH) in the ewe. A single iv injection of L-dopa (7 mg/kg) into ewes on day 3 of the estrous cycle failed to elicit an LH release during the 32-hr sampling period in three of four animals. In one ewe, however, elevated serum LH concentrations of 19.6 and 91.3 ng/ml were detected at 24 and 32 hr post-injection of L-dopa, respectively. A single im injection of 750 μg of 17β-estradiol into ewes at the same stage of the estrous cycle caused an increase in serum LH concentration in each ewe (range, 20 to 30 ng/ml) within 16 hr following treatment. A similar injection of estradiol into ewes 8 hr prior to necropsy on day 3 of the cycle was without effect on the concentrations of biogenic amines in the stalk median eminence (SME) or hypothalamus proper (HP). In both treated and control ewes, however, norepinephrine (NE) concentrations were higher (P < .01) in the HP than in the SME. Conversely, dopamine and serotonin concentrations were found to be higher (P < .01) in the SME than in the HP. Treatment of ovariectomized ewes with 750 μg of estradiol 3 hr prior to necropsy elevated NE concentrations in the SME (P = .07) but failed to alter NE concentrations in the HP. Although not significant statistically, serotonin concentrations in both the SME and HP tended to increase after treatment of these ewes with estradiol. One hour after iv injection of ovariectomized ewes with L-dopa (12 mg/kg) biogenic amine concentrations in the SME and HP qualitatively resembled those detected in ewes following injection of estradiol.

Introduction

In the rat (Brown-Grant et al., 1970) and ewe (Scaramuzzi et al., 1970) the ovulatory surge of luteinizing hormone (LH) is preceded by an increase in the levels of circulating estrogen. Evidence indicates this proestrus increase in estrogen elicits the ensuing ovulatory surge of LH. Injection of estradiol into the ovariec-tomized rat (Callantine et al., 1966) or ewe (Scaramuzzi et al., 1971) or into the intact ewe during the early stages of the estrous cycle (Bolt et al., 1971) induces a release of LH characteristic of the ovulatory surge of this gonadotropin. It is generally accepted that estrogen acts on the hypothalamo-hypophysial axis to elicit the release of LH but the mode of action of this steroid at these sites is unknown.

The ability of endogenous or exogenous estrogen to induce the release of LH may be mediated in part via the hypothalamic biogenic

1 Technical Paper No. 3623, Oregon Agricultural Experiment Station.
2 This investigation was supported by a special grant (No. 016-15-03) from the Cooperative State Research Service, U.S.D.A.
3 Appreciation is expressed to the Endocrinology Study Section, National Institutes of Health for the gift of NIH-LH-S17; Dr. G.D. Niswender for the anti-ovine LH serum and Dr. L.E. Reichert for the highly purified ovine LH (LER-1056-C2) which were used for LH radioimmunoassay.
4 Department of Animal Science.
amines. Evidence derived from investigations with laboratory animals demonstrates that the hypothalamic biogenic amines norepinephrine (NE), dopamine (DA) and serotonin (5-HT) play a prominent role in regulating the secretion of neurohormones which affect the release of gonadotropins (Kordon and Glowinski, 1972). Altering the concentrations of hypothalamic biogenic amines in the rat through the administration of the catecholamine precursor, L-dihydroxyphenylalanine (L-dopa), can cause a release of LH (Gay, 1972; Dickey, 1971). Furthermore, concentrations of hypothalamic biogenic amines are markedly altered in the rat (Donoso and Stefano, 1967) and ewe (Wheaton et al., 1972) immediately prior to the ovulatory surge of LH when plasma estrogen levels are elevated.

In the present experiments, the ability of exogenous estradiol to induce a release of LH in the ewe was used as a model to investigate the effects of this steroid on hypothalamic biogenic amine concentrations. In addition, the effect of L-dopa on hypothalamic biogenic amine concentrations and LH release in the ewe was studied.

Materials and Methods

Experimental Animals. Three experiments were conducted with 2-year-old crossbred ewes ranging in weight from 53 to 64 kilograms. The length of the estrous cycle of these ewes averaged 16 days with the first day of detected estrus designated as day 0 of the cycle. Ewes were penned with vasectomized marker rams and checked for estrus in the morning and evening of each day.

Experiment 1. Eleven ewes were assigned randomly to one of three groups. In group 1, estradiol (750 µg) dissolved in 1 ml of corn oil was injected intramuscularly (im) into four ewes. In group 2, four ewes were injected via the jugular vein with a single dose of L-dopa (7 mg/kg) dissolved in warm .01 N HCl (8 mg/ml) immediately prior to use. In the third group, one ewe was injected im with corn oil and two animals received an iv injection of .01 N HCl (8 mg/ml) immediately prior to use. In the third group, one ewe was injected im with corn oil and two animals received an iv injection of .01 N HCl (8 mg/ml) immediately prior to use. In the third group, one ewe was injected im with corn oil and two animals received an iv injection of .01 N HCl (8 mg/ml) immediately prior to use. In the third group, one ewe was injected im with corn oil and two animals received an iv injection of .01 N HCl (8 mg/ml) immediately prior to use.

Experiment 2. Ten ewes were assigned randomly in equal numbers to a treatment or control group. Treatment consisted of an im injection of 750 µg of 17β-estradiol at 2400 hr on day 2 of the estrous cycle. Control ewes received a similar injection of corn oil only. All ewes were sacrificed by exsanguination at 0800 hr the next morning. At the abattoir the brain was exposed and the stalk median eminence (SME) and hypothalamus proper (HP) were excised. The SME was severed from the pituitary and HP, exposing the opening to the third ventricle. The HP was defined rostrally by the posterior limit of the optic chiasm, 5 mm laterally from each side of the opening to the third ventricle, posteriorly by the anterior border of the mammillary body and dorsally by a depth of 10 millimeters. Mean wet weight of the SME and HP were 23 and 291 mg, respectively. Approximately 5 min elapsed from the time of exsanguination until the brain tissues were excised and packed in ice; another 15 min passed before the tissues were subjected to biogenic amine analysis which was completed the same day. Data were analyzed statistically using Student’s unpaired and paired t tests for treatment and brain tissue comparisons, respectively.

Experiment 3. Eighteen ewes were bilaterally ovariectomized and allowed a 7-week recovery period before being assigned randomly in equal numbers to three groups. The following treatments were imposed: 750 µg of 17β-estradiol in corn oil injected intramuscularly (six ewes); L-dopa, injected iv at a dose of 12 mg/kg dissolved in .01 N HCl (six ewes); or corn oil or .01 N HCl appropriately injected into three ewes each. Estradiol was injected into ewes 3 hr prior to necropsy since a study with ovariectomized rats demonstrated that significant changes in midbrain biogenic amine concentrations occurred within 3 hr following estradiol injection (Tonge and Greengrass, 1971). L-dopa was injected 1 hr before sacrifice. All ewes were killed by exsanguination at 0800 hr and the same experimental procedures
for excising and assaying the SME and HP for biogenic amines were followed as described in experiment 2. Treatments were assigned to pairs of animals to permit day of assay to be blocked in a balanced incomplete block design with three replications (Cochran and Cox, 1957). Data were analyzed statistically using least squares analysis of variance.

**Biogenic Amine Assay.** The protocol for the simultaneous assay of NE, DA and 5-HT reported by Shellenberger and Gordon (1971) was followed with slight modification. Briefly, the SME and HP were homogenized in perchloric acid solution, centrifuged and the supernatant adjusted to pH 7.8 with tricine-EDTA buffer solution which facilitates catecholamine retention on alumina. Instead of accomplishing the pH adjustment as suggested by these investigators, each sample was adjusted with buffer using a pH meter equipped with a micro-electrode. This procedure was adopted because in our laboratory the tricine-EDTA buffer solution did not possess the same pH characteristics (pH 12.5 at room temperature) ascribed to it by Shellenberger and Gordon. Catecholamines were eluted from alumina with acid and oxidized with iodine to form fluorescent derivatives which were identified by their characteristic activation and fluorescent wavelengths. Indoleamines were separated by solvent extraction with heptanol and phosphate buffer and oxidized with ninhydrin to form fluorescent derivatives. Fluorescent derivatives of NE, DA and 5-HT were measured using an Aminco-Bowman Spectrophotofluorometer. In order to quantify the assay, the HP was homogenized in 5 ml and divided into 5 aliquots, each adjusted to 3 ml with perchloric acid solution and utilized as follows: HP sample, HP tissue blank and HP plus three internal standards containing 25, 50 or 100 ng (free base) each of norepinephrine, dopamine hydrochloride and serotonin creatinine sulfate. Due to the small size of the SME, the HP tissue blank was also used as the tissue blank for the SME.

In experiment 3, to ensure an adequate amount of tissue for assay purposes, the larger HP of the pair of ewes necropsied on each day was divided into aliquots for the tissue blank as well as the internal standards. Dopamine concentrations in the SME and HP of ewes in the third experiment are not presented due to fluorescent interference by exogenous L-dopa in the assay of DA. Normally, endogenous concentrations of L-dopa are negligible alleviating interference by this catechol. By chance the HP from the L-dopa treated ewes was frequently used as the source of tissue for the blank and standard tubes. Consequently a large part of the data on DA concentrations in the SME and HP of control and estradiol-treated ewes, like that of ewes injected with L-dopa, were exceedingly high and variable.

**LH Radioimmunoassay.** Blood samples were allowed to clot at room temperature and then refrigerated. Serum was separated by centrifugation and frozen for subsequent LH assay by the radioimmunoassay protocol established and validated by Niswender et al. (1969). Highly purified ovine LH (LER-1056-C2) was iodinated with $^{125}$I and plasma LH was expressed in terms of an ovine LH standard (NIH-LH-S17). Ovine anti-rabbit gamma globulin prepared in this laboratory was used to precipitate the antisera to LH.

**Results**

**Experiment 1.** Treatment of ewes in group 1 with 750 µg of estradiol on day 3 of the estrous cycle was followed by a release of LH not later than 16 hr after injection in each of the four animals (figure 1). Characteristics of the induced LH release such as duration, magnitude and the interval of time between injection and release resembled those of the LH release in ewes in a similar experiment (Bolt et al., 1971) and in estradiol-treated anestrous ewes (Goding et al., 1969). Three of the four ewes (0290, 0314, 0362) treated with estradiol were found to have new corpora lutea when examined.

A single injection of L-dopa (7 mg/kg) into ewes on day 3 of the estrous cycle failed to elicit an LH release during the 32-hr sampling period in three of the four animals (figure 1). Luteinizing hormone concentrations in these three ewes were not different from those receiving vehicle which had an average serum LH concentration of 3.2 ng/ml. Ewe 0575 was an exception; elevated serum LH concentrations of 19.6 and 91.3 ng/ml were detected at 24 and 32 hr post-injection, respectively. The time of response relative to the injection of L-dopa, the double release pattern and the magnitude of the release were not indicative of
Experiment 2. Concentrations of biogenic amines detected in the SME and HP of estradiol-treated and control ewes on day 3 of the estrous cycle are presented in table 1. No statistically significant changes in biogenic amine concentrations were found in the SME or HP of ewes 8 hr after injection of estradiol. In all cases, however, biogenic amine concentrations in the SME were significantly different (P < .01) from those detected in the HP. The NE concentrations were lower in the SME than in the HP but concentrations of DA and 5-HT in the SME were greater than the concentrations of these amines in the HP.

Experiment 3. Concentrations of biogenic amines in the SME and HP of ovariectomized ewes 3 hr following estradiol administration are presented in table 2. Exogenous estradiol elevated NE concentrations in the SME by over 100% (P ≈ .07) but failed to affect NE concentrations in the HP. Treatment of ewes with L-dopa did not result in significant changes in the NE concentrations in the SME or HP. Substantial quantities of 5-HT were detected in the SME as compared to the HP of ovariectomized ewes and the metabolism of this monoamine appears to be sensitive to both treatment with estradiol or L-dopa. Although not significant statistically, treatment of ewes with estradiol was followed by a 50% increase in 5-HT concentrations in the SME and HP while L-dopa increased the SME and HP 5-HT concentrations approximately 25% over those in control ewes. This same trend in hypothalamic 5-HT concentrations has been noted in the rat following injection of estradiol (Tonge and Greengrass, 1971) or L-dopa (Hyypälä et al., 1971).

Discussion

Exogenous estradiol was capable of inducing a release of LH on day 3 of the estrous cycle in each of the ewes treated. A similar injection of estradiol into ewes at this stage of the cycle was without effect on the concentrations of biogenic amines in the SME and HP when measured 8 hr after injection. Perhaps estrogen evokes the spike release of LH by acting directly on the neurosecretory cells elaborating luteinizing hormone-releasing hormone (LRH) and/or by sensitizing the anterior pituitary to LRH. In this connection, the presence of estrogen has been demonstrated to facilitate the release of LH following the injection of synthetic LRH/FRH into the rat (Arimura and Schally, 1971) and ewe (Reeves et al., 1971). Similarly, treatment of rats with L-dopa resulted in a release of LH only when the drug was administered during proestrus (Gay, 1972) or following ovariectomy and pretreatment with estradiol (Dickey, 1971). Accordingly, the release of LH detected in one of the four ewes injected on day 3 of the estrous cycle with L-dopa may have been facilitated by an unusual

an estrogen-induced release. Upon ovarian examination 3 days after treatment a 4- to 5-day old corpus luteum was observed in ewe 0575 which eliminated the possibility of a delayed spontaneous ovulatory LH surge. None of the ewes treated with L-dopa were found to have new corpora lutea.

Experiment 2. Concentrations of biogenic amines detected in the SME and HP of estradiol-treated and control ewes on day 3 of the estrous cycle are presented in table 1. No statistically significant changes in biogenic amine concentrations were found in the SME or HP of ewes 8 hr after injection of estradiol. In all cases, however, biogenic amine concentrations in the SME were significantly different (P < .01) from those detected in the HP. The NE concentrations were lower in the SME than in the HP but concentrations of DA and 5-HT in the SME were greater than the concentrations of these amines in the HP.

Experiment 3. Concentrations of biogenic amines in the SME and HP of ovariectomized ewes 3 hr following estradiol administration are presented in table 2. Exogenous estradiol elevated NE concentrations in the SME by over 100% (P ≈ .07) but failed to affect NE concentrations in the HP. Treatment of ewes with L-dopa did not result in significant changes in the NE concentrations in the SME or HP. Substantial quantities of 5-HT were detected in the SME as compared to the HP of ovariectomized ewes and the metabolism of this monoamine appears to be sensitive to both treatment with estradiol or L-dopa. Although not significant statistically, treatment of ewes with estradiol was followed by a 50% increase in 5-HT concentrations in the SME and HP while L-dopa increased the SME and HP 5-HT concentrations approximately 25% over those in control ewes. This same trend in hypothalamic 5-HT concentrations has been noted in the rat following injection of estradiol (Tonge and Greengrass, 1971) or L-dopa (Hyypälä et al., 1971).

Discussion

Exogenous estradiol was capable of inducing a release of LH on day 3 of the estrous cycle in each of the ewes treated. A similar injection of estradiol into ewes at this stage of the cycle was without effect on the concentrations of biogenic amines in the SME and HP when measured 8 hr after injection. Perhaps estrogen evokes the spike release of LH by acting directly on the neurosecretory cells elaborating luteinizing hormone-releasing hormone (LRH) and/or by sensitizing the anterior pituitary to LRH. In this connection, the presence of estrogen has been demonstrated to facilitate the release of LH following the injection of synthetic LRH/FRH into the rat (Arimura and Schally, 1971) and ewe (Reeves et al., 1971). Similarly, treatment of rats with L-dopa resulted in a release of LH only when the drug was administered during proestrus (Gay, 1972) or following ovariectomy and pretreatment with estradiol (Dickey, 1971). Accordingly, the release of LH detected in one of the four ewes injected on day 3 of the estrous cycle with L-dopa may have been facilitated by an unusual
TABLE 1. CONCENTRATIONS OF BIOGENIC AMINES IN THE STALK MEDIAN EMINENCE (SME) AND HYPOTHALAMUS PROPER (HP) OF INTACT EWES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biogenic amine (µg/g wet tissue weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Norepinephrine&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SME</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>.79 ± .23</td>
</tr>
<tr>
<td>Controls</td>
<td>1.12 ± .25</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SE of five ewes per group. Ewes were injected intramuscularly with 750 µg 17β-estradiol or corn oil 8 hr prior to necropsy at 0800 hr on day 3 of the estrous cycle.<br><sup>b,c,d</sup>SME vs HP, P < .01.

It is conceivable that the temporal aspects of the second experiment may not have been optimal for the detection of estradiol induced changes in biogenic amine concentrations in the SME and HP. Eight hours after injection of estradiol was selected as the time of sacrifice because existing data indicated that LH levels generally rise in ewes shortly after this time interval (Bolt et al., 1971). If estradiol-induced changes in the SME or HP biogenic amine concentrations responsible for triggering LH release are short lived, then the variation in time from injection of steroid to the onset of LH release could account for the inability to detect significant changes in amine concentration in these hypophysiotropic areas at a constant time of necropsy.

The results of the third experiment demonstrate that an injection of estradiol into ovariectomized ewes induced changes, though not significant statistically, in SME and HP biogenic amine concentrations 3 hr after treatment. Similar changes in the concentrations of NE and 5-HT have been reported to occur in the mid-brain of estrogen-treated-ovariectomized rats 3 hr post-injection (Tonge and Greengrass, 1971).

Estrogen induced changes in biogenic amine concentrations in the central nervous system of the ovariectomized ewe, particularly in the SME, suggest that this steroid may act at least in part via monoaminergic systems to alter gonadotropin secretion in these animals. It is difficult to evaluate the relationship between the estradiol induced changes in hypophysiotropic biogenic amines and LH secretion in the ovariectomized ewe. A depression of the elevated serum LH levels in the ovariectomized ewe occurs immediately following a single injection of estradiol (Scaramuzzi et al., 1971). This depression in serum LH persists until the onset of the estradiol induced spike release of this gonadotropin. Changes in NE and 5-HT concentrations in the SME and HP in the ovariectomized ewe after the injection of estradiol may be related to the concomitant decrease in serum LH concentrations induced by this steroid.

Injection of L-dopa into ovariectomized ewes

TABLE 2. CONCENTRATIONS OF BIOGENIC AMINES IN THE STALK MEDIAN EMINENCE (SME) AND HYPOTHALAMUS PROPER (HP) OF OVARIECTOMIZED EWES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biogenic amine (µg/g wet tissue weight)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Norepinephrine</td>
</tr>
<tr>
<td></td>
<td>SME</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>.85 ± .12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-dihydroxyphenylalanine (L-Dopa)</td>
<td>.67 ± .12</td>
</tr>
<tr>
<td>Controls</td>
<td>.37 ± .12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means (based on six ewes per group) were adjusted for experimental incomplete blocks. Standard errors were calculated using a common estimate of variance. Ovariectomized ewes were either injected intramuscularly with 750 µg of 17β-estradiol 3 hr prior to necropsy; injected intravenously with L-dopa (12 mg/kg) 1 hr before necropsy or injected with vehicle only. All ewes were necropsied at 0800 hour.<br><sup>b</sup>Estradiol vs controls, P ≥ .07.
ewes 1 hr prior to necropsy resulted in qualitative changes in the concentrations of NE and 5-HT in the SME and HP similar to those in ewes treated with estradiol. Both NE and 5-HT concentrations in the SME tended to increase while only the level of 5-HT increased in the HP. The manner by which L-dopa affects 5-HT metabolism is not understood. Other investigations have demonstrated a relationship between this catecholamine precursor and indoleamine metabolism (Hyyppä et al., 1971).

Highly significant differences were detected between the concentrations of biogenic amines present in the SME and HP of ewes. Dopamine is present in high concentrations in the SME yet almost undetectable in the HP while NE concentrations are low in the SME and high in the HP. These differences in concentrations of catecholamines between the hypothalamus and median eminence of the ewe are similar to those reported to exist between the hypothalamus and median eminence of the rat as determined histochemically (Jonsson et al., 1971). Serotonin-containing nerve fibers have not yet been histochemically detected in the infundibular area. In the ewe, the concentration of 5-HT, as determined through chemical analysis, is greater in the SME than in the HP. Substantial concentrations of this indoleamine have also been chemically isolated from the SME of the bovine (Piezzi et al., 1970) and rat (Clementi et al., 1970) which suggests that the region of the median eminence receives considerable serotoninergic innervation in these species. The observed differences in the biogenic amine concentrations between the SME and HP of the ewe demonstrate the advantage of independent chemical analysis of these hypothalamic areas for amines.

Literature Cited


Hyyppä, M., P. Lehtinen and U. K. Rinne, 1971. Serotonin-containing nerve fibers have not yet been histochemically detected in the infundibular area. In the ewe, the concentration of 5-HT, as determined through chemical analysis, is greater in the SME than in the HP. Substantial concentrations of this indoleamine have also been chemically isolated from the SME of the bovine (Piezzi et al., 1970) and rat (Clementi et al., 1970) which suggests that the region of the median eminence receives considerable serotoninergic innervation in these species. The observed differences in the biogenic amine concentrations between the SME and HP of the ewe demonstrate the advantage of independent chemical analysis of these hypothalamic areas for amines.