Estradiol alters cholecystokinin stimulus-response coupling in rat pancreatic acini

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IT HAS BEEN RECOGNIZED for some time (10) that the integrity of the exocrine pancreas may be linked to estrogen status. Estrogen replacement therapy is common during menopause and subsequent to bilateral oophorectomy (8, 29), and estrogens are also administered for contraceptive purposes (9). Evidence exists that suggests that patients administered estrogens experience an increased risk of pancreatitis (7, 26).

Studies from this laboratory and others have demonstrated that estrogens play a physiological role in the exocrine pancreas, influencing both structure and function (1, 2, 14). A significant decline in pancreatic protein content, marked depletion of zymogen granules, and widening of peri- and interlobular spaces are associated with simultaneous ovariectomy and adrenalectomy of the female rat, and a single 1-mg intraperitoneal injection of estradiol results in restoration of zymogen granules and a normal appearance within 9 h (1). Acute hemorrhagic pancreatic necrosis can be induced in female mice fed a choline-deficient diet; however, in addition to being fed this diet, male mice must also be treated with estradiol to induce pancreatic necrosis (23).

These observations suggest that estrogens exert substantial influence over exocrine pancreatic function. There is, however, a paucity of information regarding the mechanisms through which estradiol influences hormone-stimulated exocrine pancreatic secretion. We have previously reported studies in which we found that chronic in vivo estradiol treatment alters pancreatic amylase secretion and CCK receptor binding characteristics (2). The underlying hypothesis of the present study was that CCK-stimulated secretory function of isolated rat pancreatic acini would be modified by chronic treatment of rats with estradiol and that these modifications would be mediated by alterations in the CCK signal transduction pathway. This hypothesis was tested utilizing doses of estradiol spanning the physiological range and measuring the effects on acinar cell amylase content, CCK-stimulated amylase secretion, CCK receptor binding characteristics, and expression of the α-subunit of the heterotrimeric G protein Gq/11 (Gq/11).

Materials and Methods

Materials. The following were purchased: collagenase from Worthington Biochem (Freehold, NJ), synthetic cholecystokinin octapeptide (CCK-8) from Peninsula Laboratories (Belmont, CA), CCK-8 labeled by Bolton-Hunter reagent method (125I-labeled BH-CCK-8, specific activity 2,000 Ci/mmol) from Amersham (Arlington Heights, IL), Eagle’s medium amino acid supplement from GIBCO (Grand Island, NY), 21-day timed-release pellets containing vehicle or estradiol from Innovative Research of America (Sarasota, FL), procion yellow dye MX-8G from Polysciences (Warrington, PA), and estradiol double antibody kit from Diagnostic Products (Los Angeles, CA). Unless otherwise noted all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Animals. The Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences approved this study, and the study was performed in accordance with the Guide for the Care and Use of Laboratory Animals.
with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Female Sprague-Dawley rats obtained from the Charles River breeding colony were housed in plastic cages with wire covers, in a temperature-controlled room (22 ± 2°C), on a 12:12-h light-dark cycle (lights on at 6 AM and off at 6 PM), and allowed ad libitum access to standard laboratory rat chow. On day 1 of the study, the respective surgery was performed and 21-day timed-release pellets containing vehicle or estradiol were implanted subcutaneously. Ovariectomized rats were administered either vehicle or estradiol at one of three doses (0.5, 24, or 119 µg/day), whereas sham ovariectomized rats were administered vehicle. On day 18 rats fasted for 18 h were killed by decapitation. Blood was removed by exanguination using heparinized tubes and centrifuged at 1,500 g for 10 min, and plasma was saved frozen at −80°C for later determination of plasma estradiol. Pancreata and uteri were removed, and wet weights were measured. Sham ovariectomized rats were utilized at random times during the estrous cycle.

Preparation of isolated pancreatic acini and amylase release. Subsequent to removal the pancreas was trimmed free of fat and lymph nodes, and enzymatic digestion was used to prepare isolated pancreatic acini as described previously (2). Acini were preincubated and resuspended in 10% HEPES-buffered Ringer solution (pH 7.4), supplemented with 11.1 mM glucose, minimum Eagle’s medium amino acid solution, 0.5% bovine serum albumin, and 0.02% soybean trypsin inhibitor. Aliquots (3 ml) of acinar suspension were incubated with various concentrations of CCK-8 for 30 min at 37°C with shaking at 60 cycles/min and 100% O2 gas phase. Duplicate flasks were used to determine stimulated amylase release for each concentration of CCK.

Preparation of enriched acinar cell membranes for 125I-labeled BH-CCK-8 binding. Pancreatic acinar cell membranes were prepared from aliquots of acini obtained after the preincubation step of acinar cell isolation. Acini were kept on ice at 4°C during all steps of membrane preparation. Acini were washed in nine volumes of buffer B (0.2 M sucrose, 10 mM HEPES, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, at pH 7.4) and sedimented by centrifugation at 300 g for 5 min. The supernatant was removed by suction and discarded, and the washing and centrifugation steps were repeated once more. The pellet obtained was then diluted with nine volumes of buffer A (1.35 M sucrose, 10 mM HEPES, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) and homogenized using six 6-s bursts of a sonic disruptor, with a 1-cm tip at a setting of 40. The homogenate was then decanted into 14 × 95 mm centrifuge tubes and buffer B was layered over the top. These tubes were centrifuged at 100,000 g for 120 min. The membrane fraction at the interface between the two layers was collected. These membranes were diluted fivefold with buffer C (20 mM HEPES, 5 mM MgCl2, 1 mM EGTA, 100 mM NaCl, at pH 6.5) and centrifuged at 30,000 g for 35 min. The pellet of enriched membranes was resuspended in buffer C, and a small aliquot was removed for measurement of protein concentration. The remainder was dissolved in gel-loading buffer for immunoblot analysis and stored at −80°C until used.

Immunoblot measurement of Goα11 expression. One dimensional gel electrophoresis was performed as described by Laemmli (18). Twenty micrograms of protein per sample were loaded in each lane of 12% SDS-PAGE minigels and run at 200 V. After gel electrophoresis, proteins were transferred to nitrocellulose membranes according to the method of Towbin et al. (28) using glycine-Tris-methanol transfer buffer containing 0.1% SDS to enhance the transfer from the gel to 30 V overnight. Nitrocellulose membranes were blocked with Tris-buffered saline (2.42 g Tris base, 8.0 g NaCl, pH 7.6) plus Tween 20, containing 5% bovine serum albumin for 1 h. Membranes were then washed and probed with anti-G0α11 antiserum (Dupont-NEN) at a concentration of 1:10,000 for 2 h. Membranes were washed again and then probed for 90 min with donkey anti-rabbit antibody conjugated with horseradish peroxidase. Membranes were then washed five additional times, and bands were visualized using enhanced chemiluminescence following the manufacturer’s procedures, by exposing the membranes to Dupont Reflection Autoradiography Films. For quantitation, film images were scanned using a flat bed scanner and saved as image files. Band densities were quantitated using the Un-Scan-It gel analysis program (Silk Scientific, Orem, UT).

Assays. Amylase activity of incubation media and calibration standards (Sera Chem, Sigma Chemical) were measured using the method of Jung (15), with pronase yellow starch as substrate. Protein was determined by the method of Bradford (5). Four milliliters of 1:5 diluted Bio-Rad dye reagent were added to each sample of protein standard or unknown, and bovine serum albumin was used as standard.

Plasma estradiol was measured using a commercial radioimmunoassay kit (DoubleAntibody kit, Diagnostic Products). 125I-labeled compounds were counted using a Packard Auto-Gamma 5650 gamma counter at 70% counting efficiency.

Data analysis. All measurements were made in duplicate and are expressed as means ± SE of the average of duplicate determinations. Statistical significance was tested by analysis of variance with the appropriate multiple range test. P < 0.05 was considered statistically significant. Binding data were analyzed using the LIGAND least-squares curve fitting program of Munson and Rodbard (19).
RESULTS

Effects of estradiol administration on plasma estradiol, uterine weight, and pancreatic weight. Plasma estradiol levels of ovariectomized rats treated with either vehicle or 0.5 µg estradiol per day were below the sensitivity of the estradiol radioimmunoassay (Table 1), and for the purpose of statistical analysis the value of radioimmunoassay sensitivity (5 pg/ml) was assigned to these groups. Plasma estradiol of these two groups was significantly less than that of sham ovariectomized rats or ovariectomized rats treated with 24 or 119 µg estradiol per day. Doses of 24 and 119 µg estradiol per day yielded measurable plasma estradiol levels and mean plasma estradiol of rats receiving 119 µg estradiol per day was significantly greater than any of the other groups.

Ovariectomy resulted in a significant \((P < 0.05)\) decrease in uterine weight compared with that of sham ovariectomized rats, and chronic estradiol treatment of ovariectomized rats was associated with an apparent dose-dependent increase in uterine weights (Table 1). No significant differences in pancreatic weights were found between any of the groups examined.

Estradiol influence on amylase content and release from exocrine pancreas. The cellular amylase content of acini isolated from estradiol-treated ovariectomized rats appeared to increase in a dose-dependent manner (Fig. 1). Cellular amylase content was significantly \((P < 0.01)\) increased by 119 µg estradiol per day, compared with acini isolated from any of the other three groups. There appeared to be a trend toward dose-dependent increases in basal amylase release in ovariectomized rats treated with estradiol, but no significant differences were observed (data not shown).

Chronic treatment of rats with estradiol was associated with apparent dose-dependent decreases in the magnitude of percent initial amylase release from acini in response to graded doses of CCK-8 (Fig. 2). The maximal percentage of initial cellular amylase released by acini isolated from ovariectomized rats was 34.4 ± 1.9%. Maximal release was decreased to 31.2 ± 4.1% by 0.5 µg estradiol per day, to 23.3 ± 3.4% by 24 µg estradiol per day, and to 9.4 ± 1.6% by 119 µg estradiol per day. Estradiol at a daily dose of 24 µg significantly decreased percent initial amylase release at doses of CCK-8 ranging from 10 to 300 pM, compared with ovariectomized rats treated with either vehicle or 0.5 µg estradiol per day. Percent initial amylase release was significantly decreased by 119 µg estradiol per day, at doses of CCK-8 ranging from 3 pM to 1 nM, compared with all other groups.

Effects of estradiol on CCK receptor binding. The affinity of the CCK receptor on pancreatic acinar cell membranes was not significantly affected by estradiol treatment of ovariectomized rats (Table 2). However, CCK receptor capacity was significantly greater on membranes from vehicle-treated ovariectomized rats compared with ovariectomized rats treated with 24 or 119 µg estradiol per day.

### Table 1. Effects of ovariectomy and estradiol on plasma estradiol, uterine, and pancreatic weights of rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma E2 (pg/ml)</th>
<th>Uterine Wt (mg)</th>
<th>Pancreatic Wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham veh</td>
<td>25.3 ± 1.3*</td>
<td>351 ± 55*</td>
<td>1.120 ± 55</td>
</tr>
<tr>
<td>Ovx veh</td>
<td>&lt;5</td>
<td>102 ± 6</td>
<td>1.153 ± 40</td>
</tr>
<tr>
<td>Ovx+ 0.5 µg E2/day</td>
<td>&lt;5</td>
<td>211 ± 16</td>
<td>1.140 ± 29</td>
</tr>
<tr>
<td>Ovx+ 24 µg E2/day</td>
<td>188 ± 62</td>
<td>322 ± 55‡</td>
<td>1.141 ± 61</td>
</tr>
<tr>
<td>Ovx+ 119 µg E2/day</td>
<td>613 ± 140†</td>
<td>772 ± 60‡</td>
<td>1.196 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of measurements. E2, estradiol; Ovx, ovariectomized; veh, vehicle. Eighteen days after surgery and initiation of estradiol treatment, plasma samples were collected, uteri and pancreata were removed, and wet weight was measured. Commercial radioimmunoassay kit was used to measure plasma estradiol. *Significantly \((P < 0.05)\) greater than Ovx veh. †Significantly \((P < 0.05)\) greater than Ovx, and Ovx+ 0.5 µg E2/day. ‡Significantly \((P < 0.05)\) greater than all other groups.

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**Fig. 1.** Total cellular amylase content of pancreatic acini isolated from sham ovariectomized (Sham), ovariectomized (Ovx), and estradiol (E2)-treated ovariectomized rats. Rats were implanted with 21-day time-release pellets that yielded the daily doses indicated. After 18 days rats were killed, pancreata were removed, acini were isolated, and CCK-stimulated amylase release was measured. *Significantly \((P < 0.05)\) greater than all other groups.

**Fig. 2.** Amylase release expressed as a percentage of initial cellular amylase content from pancreatic acinar cells isolated from sham ovariectomized (S-Ovx), ovariectomized, and estradiol-treated ovariectomized rats. Veh, vehicle. *Significantly \((P < 0.05)\) less than Ovx veh. **Significantly \((P < 0.05)\) less than Ovx veh and 0.5 µg E2. ***Significantly \((P < 0.05)\) less than all other groups.
Table 2. Effect of estradiol administration on affinities and capacities of CCK receptors on pancreatic acinar cell membranes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>K_d (pM)</th>
<th>B_max (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham Ovx + veh</td>
<td>6</td>
<td>62.6 ± 5.9</td>
<td>373.8 ± 26.2*</td>
</tr>
<tr>
<td>Ovx + veh</td>
<td>8</td>
<td>51.8 ± 3.6</td>
<td>1,051.6 ± 115.5</td>
</tr>
<tr>
<td>Ovx + 0.5 µg E_2/day</td>
<td>6</td>
<td>55.0 ± 10.0</td>
<td>791.8 ± 153.6</td>
</tr>
<tr>
<td>Ovx + 24 µg E_2/day</td>
<td>5</td>
<td>55.2 ± 4.6</td>
<td>405.5 ± 42.2*</td>
</tr>
<tr>
<td>Ovx + 119 µg E_2/day</td>
<td>6</td>
<td>67.9 ± 10.3</td>
<td>424.4 ± 107.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. rats. PANNEAUD acinar cell membranes were incubated with 7 pM [125I]-labeled BH-CCK-8 and doses of synthetic CCK-8 ranging from 10 pM to 100 nM. At 150 min, incubation mixture was filtered and washed, and bound radioactivity was determined. Equilibrium dissociation constant (K_d) and maximal binding capacity (B_max) were determined using LIGAND nonlinear curve-fitting program. *Significantly (P < 0.01) less than Ovx + veh.

Several possible short-term effects of estradiol were observed. To determine whether the effects of estradiol could be observed over the short term, estradiol was included in the incubation medium along with 100 pM CCK, at concentrations between 1 pM and 100 nM. In this acute study no apparent effect of estradiol was observed on CCK-8-stimulated amylase release or total CCK binding (data not shown).

Examination of possible short-term estrogen effects on the exocrine pancreas. Ovariectomy had no significant effect on pancreatic Gq/11 expression compared with sham ovariectomized rats (data not shown). However, treatment of ovariectomized rats with increasing concentrations of estradiol was associated with significant (P < 0.001) increases in Gq/11 expression (Fig. 3).

**DISCUSSION**

We have previously observed that chronic ovariec-

omy is associated with increased sensitivity of the exocrine pancreas to CCK stimulation and increased CCK receptors on pancreatic acinar cell membranes, whereas a dose of estradiol in the upper physiological range was associated with both suppression of the magnitude of CCK-stimulated amylase release and a decrease in the number of CCK receptors (2). The present study examined the influence of chronic estrogen deficiency induced by ovariectomy and chronic exposure to a range of doses of estradiol on pancreatic acinar cell amylase content, CCK-stimulated amylase secretion, CCK receptor binding characteristics, and expression of Gq/11. Plasma estradiol levels were also measured, and uterine weights of ovariectomized rats treated with vehicle or estradiol were used to assess the bioactivity of the estradiol preparation employed in these studies (14, 21). Plasma estradiol of the rats administered the two highest doses attained levels found during pregnancy and in individuals receiving very high doses of estradiol (27).

The dose-dependent increases in total cellular amylase associated with chronic estradiol administration are likely the result of increases in either synthesis or storage of amylase. The decreased responsiveness of acini from estradiol-treated rats suggests that the increased total cellular amylase is probably due to increased intracellular amylase stores resulting from decreased stimulated secretion. The present study did not directly address the question of estradiol effects on amylase synthesis.

In the present study we found that chronic administration of estradiol to ovariectomized rats was associated with dose-dependent decreases in the magnitude of CCK-stimulated amylase release. Guo and Singh (13) found that total stimulated amylase release was significantly decreased when ovariectomized guinea pigs were administered daily injection of estradiol. This finding indicates that chronic estradiol administration decreases the responsiveness of CCK stimulus-response coupling in pancreatic acini.

Recently, there has been increased interest in the nongenomic effects of steroid hormones, and membrane-bound binding sites for estrogens have been found in the brain (6). Whether such receptors exist in the exocrine pancreas is not presently known. However, pancreatic tissue has a great capacity to retain estrogen.

**Fig. 3.** A: representative immunoblot of enriched rat pancreatic membranes from ovariectomized rats chronically treated with increasing doses of estradiol. Blot was probed with antisera raised against Gq/11. B: pooled data of Gq/11 band densities, measured by scanning and densitometry using the Un-Scan-it program (Silk Scientific n = 5). *Significantly (P < 0.01) greater than 0 or 0.5 µg estradiol/day.
The observed estrogen-induced alteration of CCK receptor binding characteristics is one mechanism that may at least partially account for the altered responsiveness of acini to CCK. CCK receptor affinity was not significantly altered by either ovariectomy or ovariec-tomy plus estradiol. We have previously shown that pancreatic membranes prepared from ovariectomized rats exhibit a significantly greater number of CCK receptors than those of sham ovariectomized rats (2). The findings of the present study show that while ovarian insufficiency is associated with increased acinar cell CCK receptor capacity, chronic estrogen administration is associated with dose-dependent decreases in CCK receptor capacity. Increased CCK binding capacity on membranes prepared from acini isolated from ovariectomized rats parallels increased responsiveness of pancreatic acini to CCK stimulation, whereas decreased CCK binding capacity on membranes prepared from estradiol-treated rats dose dependently parallels decreases in responsiveness of pancreatic acini to CCK-8. It is likely that the altered responsiveness to CCK-8 of pancreatic acini isolated from ovariectomized rats is at least partially the consequence of alterations in CCK receptor numbers. The mechanisms through which estrogens induce these effects on pancreatic CCK receptors are not clear.

Because the CCK receptor on acinar cells exists in multiple interconvertible binding affinity states (3), it is possible that estrogens may influence the affinity state of the receptor. It is also possible that chronic estradiol administration may be associated with altered receptor internalization or insulation (24). Although the most dramatic decrease in CCK-8-stimulated amylase release was observed in rats treated with 119 µg estradiol per day, there was no further decrease in the number of CCK receptors between that group and the 24 µg estradiol per day group, suggesting that alterations in stimulus-secretion coupling downstream of the CCK receptor may also play a role in the effects observed at this highest dose of estradiol.

The mechanism through which binding of CCK to its receptor leads to activation of phospholipase C is believed to be via the interaction of the ligand bound receptor with a guanine nucleotide binding protein (30). Expression of the α-subunit of Gq11, the hetero-trimeric guanine nucleotide binding protein believed to couple to the CCK receptor, was examined by Western blotting to further examine estrogen effects on the CCK signal transduction pathway as a possible mechanism mediating the alterations in secretion. Expression of Gq11 was dose dependently increased in acinar cell membranes from ovariectomized rats treated with estradiol. Recently it has been found that Gq11 is localized on pancreatic zymogen granules and involved in calcium-regulated amylase secretion (20). Ohnishi et al. (20), utilizing a known antagonist of Gq11, demonstrated that antagonism of Gq11 was associated with a concentration-dependent potentiation of calcium-stimulated amylase release. The authors concluded that Gq11 on zymogen granules plays a tonic inhibitory role in calcium-regulated amylase secretion from pancreatic acini. In view of these findings, the dose-dependent increases in the expression of Gq11 in estradiol-treated rats may be involved in the inhibition of CCK-stimulated amylase release from estradiol-treated acini. It is possible that estrogens also influence stimulus-secretion coupling at other points in the CCK signal transduction pathway or may be associated with altered receptor G protein coupling. Our present data do not rule this out but do demonstrate that the observed effects of estradiol on amylase secretion are likely mediated by alterations in CCK receptor numbers and Gq11 expression.

The most obvious manner through which estradiol might influence the expression of CCK receptors and Gq11 is via the classical estrogen receptor, acting to induce changes in transcriptional mechanisms (16). It will be interesting to determine whether the change in receptor density on acinar cell membranes and Gq11 expression is the result of modified transcriptional or translational mechanisms resulting in altered production of these proteins. Moreover, estradiol may possibly be acting through membrane-bound estradiol binding sites. The present study examined the influence of ovariectomy and estradiol administration on CCK-mediated signal transduction mechanisms. Whether the effects of estradiol are specific for the CCK signaling pathway or may influence signaling mediated by other acinar cell stimulants such as bombesin, vasoactive intestinal peptide, and acetylcholine is not known. These questions are presently under investigation.

In conclusion, we have characterized the effects of chronic estradiol administration on pancreatic acinar
cell amylase secretion stimulated by CCK. We have also demonstrated that estrogens decrease CCK receptor binding while increasing \( \text{G}_{\delta1} \) expression. These observations provide new insight for understanding the mechanisms through which estrogens influence exocrine pancreatic function.

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