Estradiol induces C-type natriuretic peptide gene expression in mouse uterus

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Acuff, Cory G., Huaming Huang, and Mark E. Steinhelper. Estradiol induces C-type natriuretic peptide gene expression in mouse uterus. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2672–H2677, 1997.—Previous experiments have demonstrated that C-type natriuretic peptide (CNP) expression in the uterus varies during the estrous cycle with maximal expression at proestrus. The present study was designed to determine whether exogenous steroid hormones regulate uterine CNP expression in ovariectomized mice. Estradiol increased significantly (3-fold) uterine immunoreactive CNP (irCNP) rapidly and dose dependently in ovariectomized mice as measured by radioimmunoassay. Other steroids produced either no significant change (testosterone, 1 mg; 2-methoxyestradiol, 1 µg) or weak induction (estradiol, 1 µg) from vehicle controls. Progesterone (1 mg) significantly attenuated the estrogen-stimulated irCNP response by 50% when injected 30 min before estrogen (1 µg) in estrogen-primed ovariectomized mice. Estrogen-stimulated increases in uterine CNP transcripts detected by ribonuclease protection analyses were blocked by actinomycin D (160 µg) or ICI-164,384 (20 µg), a specific nuclear estrogen receptor antagonist. These results indicate that a nuclear estrogen receptor is required for estrogen to stimulate uterine CNP transcription and that progesterone negatively regulates estrogen-stimulated CNP expression.

Materials and Methods

Materials. Plasmids, restriction endonucleases, and other enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), Novagen (Madison, WI), Perkin-Elmer (Norwalk, CT), Promega (Madison, WI), and Stratagene (La Jolla, CA). Radiosotopes were obtained from DuPont-NEN (Wilmington, DE). Mammalian cells were maintained in serum-free and serum-containing media, human chronic gonadotropin, human chorionic gonadotropin, steroids, and other reagents were purchased from Sigma (St. Louis, MO). Commercial reagents for CNP radioimmunoassay (RIA) were purchased from Peninsula Laboratories (Belmont, CA).

Animals. CD-1 outbred mice were obtained from Charles River (Wilmington, MA). Mice were maintained on a 12-h light/12-h dark cycle, with access to food (Prolab R-M-H 1000, Agway) and water ad libitum. Mice were killed (1000–1200 h) by cervical dislocation, and selected tissues were rapidly frozen on dry ice and stored at −80°C until use.

C-type natriuretic peptide (CNP) is encoded by a member of the natriuretic peptide multigene family whose protein products regulate hydromineral homeostasis and blood pressure. CNP was originally described as a predominantly central nervous system peptide (26); however, subsequent studies demonstrated CNP expression at discrete peripheral sites (18, 25, 27, 28), including the female reproductive system of mice and rats (12, 14). Infusion of CNP in dogs produced hypotension but without the overt natriuresis and diuresis associated with atrial natriuretic peptide or brain natriuretic peptide infusion (24). Because the concentration of circulating CNP is extremely low, CNP produced in peripheral tissues has been proposed to act predominantly through paracrine or autocrine mechanisms (25). CNP transcripts were detected in bovine carotid endothelial cells in tissue culture (25, 28) and were stimulated by transforming growth factor-β (TGF-β) or basic fibroblast growth factor but not by platelet-derived growth factor. A separate study showed that endothelial CNP secretion is regulated by interlukins, tumor necrosis factor, and lipopolysaccharide (27). Vascular endothelial growth factor (VEGF) has been shown to inhibit CNP secretion (7). These findings are consistent with the widespread expression of natriuretic peptide receptors in several peripheral tissues, including female reproductive organs, and are consistent with the widespread expression of natriuretic peptide receptors in several peripheral tissues, including female reproductive organs (4, 8, 14).

The mouse gene (Nppc) encoding CNP was isolated and mapped to chromosome 1 (12, 19), distinct from the chromosome 4 cardiac natriuretic peptide locus containing Nppb and Nppa in a tandem array (13, 29). Analysis of CNP expression in mice of both genders demonstrated that female reproductive organs (ovary and uterus) had the highest amounts of CNP transcripts and immunoreactive CNP (irCNP) of those peripheral tissues surveyed (12). Although ovarian CNP expression was relatively constant on the morning of each estrous cycle stage, uterine CNP expression was highly dynamic at both the transcript and protein level. Uterine CNP transcripts and immunoreactive protein were lowest at estrus and maximal at proestrus. CNP transcripts were not detected in the uterus from prepubertal mice.

Variation in uterine CNP expression correlated well with the known changes in uterine mass and fluid content during development and the estrous cycle. However, the physiological factors that regulate CNP expression in the uterus were not identified. Given that appropriate regulation of uterine hydromineral homeostasis likely contributes to successful mammalian reproduction, it is important to identify those factors that regulate CNP expression in the uterus. Previous studies have shown that peptide and steroid hormones comprising the hypothalamic-pituitary-gonadal axis can influence uterine hydromineral homeostasis (11). The present study was designed to identify physiological hormonal components of the hypothalamic-pituitary-gonadal axis that regulate uterine CNP expression in mice.
isolated and frozen on dry ice. Tissues were stored at -80°C until further use. All experimental procedures were approved by the Institutional Animal Care and Use Committee on the safe and humane use of experimental animals.

Ovariectomy. Female mice, anesthetized with ketamine- xylazine (10–1.5 mg/100 g body wt), received a sham surgery or were bilaterally ovariectomized. Sham animals received all incisions and organ manipulations, and the remaining animals had the ovary and proximal oviduct removed. Animals were allowed to recover from surgery for 5–7 days before further experimentation.

Effects of estrogen and other steroids on uterine CNP expression. Ovariectomized mice (n = 5) were given a single intraperitoneal injection of either 10, 30, 100, 300, or 1,000 ng/20 g body wt of 17β-estradiol-3-benzoate (E2) in 95% corn oil-5% ethanol (vehicle). Animals were killed 6 h after the injection was administered. Individual uterine horns were isolated at the point of bifurcation from the vagina to the distal portion of the oviduct. Connective tissue and uterine vessels were removed and uterine horns quickly frozen on dry ice. Frozen tissue was weighed before extraction for RIA or RNA isolation. The ratio of uterine wet weight to 20 g body weight was recorded for each animal. Uterine irCNP levels were determined by RIA. The kinetics of the uterine irCNP response to exogenous estrogen in ovariectomized mice was determined in ovariectomized mice (n = 25) injected with 1,000 ng/20 g body wt of E2 (5). Mice were killed at 1, 2, 6, and 24 h after E2 injection, and uterine horns was collected, and irCNP content of paired uterine horns was determined by RIA. Ovariectomized mice (n = 5 per treatment) were injected with either vehicle, testosterone (1 mg/20 g body wt), estradiol (1 mg/20 g body wt), or 2-methoxyestradiol (1 mg/20 g body wt). A second series was performed where ovariectomized CD-1 mice (n = 4–7 per group) were pretreated with E2 (1 mg/20 g body wt) for 2 days followed on the third day by an injection of either vehicle, E2 (1 mg/20 g body wt), progesterone (1 mg/20 g body wt), or progesterone followed 30 min later by E2 (1 mg/20 g body wt) (1). Mice were killed 6 h after the last injection, and uterine horns were removed and processed for irCNP analysis by RIA.

Inhibition of estrogen-stimulated uterine CNP transcription. Ovariectomized mice (n = 6) were intraperitoneally injected with vehicle, 1 mg/20 g body wt of ethanol or E2 (1 mg/20 g body wt). Some mice receiving E2 were previously injected with actinomycin D (160 µg/20 g body wt) to inhibit transcription. Mice were killed 2 h after the second injection, and uterine horns were removed and processed for analysis of CNP transcripts. A second group of mice (n = 9) was injected with either vehicle or ICI-164,384 (20 µg/20 g body wt) followed 2 h later by vehicle or E2 (1 mg/20 g body wt). Mice were killed 2 h after the second injection, and uterine horns were removed and processed for analysis of CNP transcripts.

RNA analyses. Total RNA was prepared by centrifugation through 5 M CsCl (3, 20) or using a commercial kit (RNAeasy, Qiagen Chatsworth, CA). Ribonuclease protection analyses were performed as previously described (20, 23), using an exon 2 antisense riboprobe generated by T3 RNA polymerase and [α-32P]UTP or [α-32P]CTP. The exon 2-specific template was a 300-base pair fragment (Apal I to BamHI I) containing a 5′-XhoI fragment isolated from a partial BALB/c genomic clone (12). A mouse glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) riboprobe was used to control for variations in RNA preparation and was produced from a commercially obtained template (Ambion, Austin, TX).

Radioimmunoassay. Frozen tissue samples used for CNP quantitation were prepared as previously described (12). Briefly, frozen tissues were boiled for 15 min in 10 volumes of acetic acid (1 M), chilled on ice, and homogenized with a Polytron (30 s). The homogenate was cleared by centrifugation, and the supernatant was applied to a Sep-pak C18 column (Millipore, Bedford, MA) previously equilibrated with 60% acetonitrile in 1% trifluoroacetic acid (TFA, 3 ml) followed by 1% TFA (3 × 3 ml). Columns were washed with 1% TFA (3 × 3 ml), and peptides were eluted from the column with 60% acetonitrile in 1% TFA (4.5 ml). Eluate was lyophilized and stored at -80°C until use. Samples were reconstituted in 0.4 ml of RIA buffer. RIA of 25–100 µl of each reconstituted sample was performed using commercial reagents (Peninsula Laboratories, Belmont, CA) according to the manufacturer’s recommendations.

Statistical analysis. Ribonuclease protection analyses results are representative of three independent assays. RIA results are expressed as mean values ± SE and analyzed using a commercial program (Prism, GraphPad, San Diego, CA) on a 586-PC. Statistical comparisons of irCNP were evaluated by Student’s unpaired t-test and one-way analysis of variance with Dunnett’s multiple comparison test. P < 0.05 was considered significant in all comparisons.

RESULTS

Previous studies implicated ovarian-derived factors as important regulators of uterine CNP expression. In the present study we investigated the sensitivity of uterine CNP gene expression to exogenous steroid hormones. As shown in Fig. 1, uterine irCNP tended to increase even at the lowest E2 dose tested (10 ng/20 g body wt). Significant increases in uterine irCNP was obtained at E2 doses of ≥30 ng/20 g body wt when compared with vehicle-injected animals. Maximal uterine irCNP content was observed between 100 and 1,000 ng/20 g body wt.

The kinetics of changes in uterine irCNP and mass after injection of E2 (1,000 ng/20 g body wt) was determined in a group of ovariectomized mice (Fig. 2). There was a significant increase in uterine irCNP at 1 h after E2 injection. As shown in Fig. 2, irCNP increased almost fourfold at 2 h after E2 injection and remained elevated two- to threefold from basal levels for at least 24 h after injection. Significant increases in uterine mass were observed after 2 h of E2 administration. Uterine mass increased to maximal levels within 6 h of

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E2 injection, which were maintained for at least 24 h (n = 8–10 in all groups). No difference in body weight was observed between groups (data not shown).

The specificity of the E2-stimulated uterine CNP response was investigated by examining the effects of other steroids at 6 h postinjection. Ovariectomized CD-1 mice were injected with either vehicle, testosterone (1 mg/20 g body wt), estradiol (1 mg/20 g body wt), or 2-methoxyestradiol (1 mg/20 g body wt). As shown in Fig. 3A, estradiol induced a very weak response, and testosterone and 2-methoxyestradiol did not increase uterine irCNP. Estradiol and testosterone, but not 2-methoxyestradiol, induced small increases in uterine mass.

It is well known that progesterone, produced predominantly by the corpus luteum after the endogenous luteinizing hormone (LH) surge, has numerous biological effects on uterine function. Accordingly, the effect of progesterone on estrogen-stimulated uterine CNP expression was examined. For this study, ovariectomized mice were pretreated for 2 days with E2 (1,000 ng/20 g body wt) to induce the expression of the progesterone receptor (9). Mice were injected on the third day with either vehicle, E2, progesterone (P, 1 mg/20 g body wt), or progesterone followed 30 min later by E2 (1,000 ng/20 g body wt) (1). IrCNP concentration was measured 6 h after the last injection. As shown in Fig. 3B, E2 administration increased uterine irCNP levels by over twofold. Progesterone injection alone failed to alter irCNP levels from vehicle-injected mice. Interestingly, pretreatment with progesterone attenuated significantly (50%) the E2-dependent increase in uterine irCNP and uterine mass.

The regulatory mechanism underlying E2-stimulated uterine CNP expression was investigated by using a specific inhibitor of transcription initiation, actinomycin D. As shown in Fig. 4A, uterine CNP transcripts were significantly elevated 2 h after E2 injection (1,000 ng/20 g body wt). This increase in CNP transcript levels was inhibited completely by administration of actinomycin D (160 µg/20 g body wt) 2 h before E2 injection. E2 has been shown to affect transcriptional regulation of various genes through its interactions with a specific nuclear receptor. The role of this receptor in E2-stimulated uterine CNP expression was investigated using a specific antagonist ICI-164,384 (33). As shown in Fig. 4B, the E2-dependent increase in CNP transcripts was inhibited by prior administration of the nuclear E2 receptor (ER) antagonist (ICI-164,384, 20 µg/20 g body wt) when injected 2 h before E2 injection (10).

**DISCUSSION**

Our previous experimental results demonstrated that uterine CNP expression varied during the estrous cycle with maximal expression at proestrus (12). A pilot study showed that bilateral ovariectomy prevented the increase in CNP mRNA and irCNP at 46 h after injection of pregnant mare serum gonadotropin into mice (data not shown). This observation, together with previous estrous cycle data (12), implicated ovarian-derived factors as important regulators of uterine CNP expression. E2 was considered the most likely candidate to regulate uterine CNP expression for several reasons.

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**Fig. 2.** Uterine irCNP and mass in ovariectomized CD-1 mice after injection of E2 (1,000 ng/20 g body wt). Tissues were isolated at times indicated and weighed, and extracts were prepared and analyzed by RIA as described in MATERIALS AND METHODS. Values are means ± SE; n = 5 mice per group. *P ≤ 0.05 compared with vehicle-injected group.

**Fig. 3.** A: specificity of E2-stimulated uterine CNP expression. Uterine irCNP and mass in ovariectomized CD-1 mice (n = 5 mice per group) after injection of vehicle, testosterone (T, 1 mg/20 g body wt), estradiol (E3; 1 mg/20 g body wt), or 2-methoxyestradiol (2ME; 1 mg/20 g body wt). Tissues were isolated 6 h after injection and weighed, and extracts were prepared and analyzed by RIA as described in MATERIALS AND METHODS. Values are means ± SE. *P ≤ 0.05 compared with vehicle-injected group (V). B: effect of progesterone on E2-stimulated uterine CNP expression. Ovariectomized CD-1 mice (n = 5–7 per group) were pretreated for 2 days with single injections of E2 (E2; 1,000 ng/20 g body wt) followed on the third day by an injection of either vehicle (V), E2, progesterone (P; 1 mg/20 g body wt), or P followed 30 min later by E2 (P + E2). Mice were killed 6 h after the last injection. Uterine horns were removed and processed for irCNP analysis by RIA. Uterine horns were isolated 6 h after the last injection and weighed, and extracts were prepared and analyzed by RIA as described in MATERIALS AND METHODS. Values are means ± SE. *P ≤ 0.05 compared with vehicle-injected group (V). #P ≤ 0.05 compared with estradiol-injected group (E2).
with either vehicle (V, n = 2) or E2 (1 µg/20 g body wt). Some mice were injected with actinomycin D (n = 2; Act-D; 160 µg/20 g body wt) 2 h before E2 injection. Mice were killed 2 h after second injection, uterine horns were isolated, and total RNA was prepared. Total RNA (10 µg) was analyzed for CNP transcripts using an exon 2-specific riboprobe. Probe and RNA were annealed at 50°C overnight and digested with RNase A and T1, and the resulting fragments were analyzed on an 8% denaturing polyacrylamide gel. Gels were fixed, dried under vacuum, and exposed to Kodak X-AR film at 80°C for 1 day. Results are representative of 3 independent experiments. B: mice were injected on the fifth day after ovariectomy with either vehicle (V) or ICI-164,384 (ICI; 20 µg/20 g body wt) at 1000 h and vehicle (V) or E2 (1,000 ng/20 g body wt) at 1200 h as indicated. Mice were killed 2 h after the second injection, uterine horns were isolated, and total RNA was prepared. Total RNA (10 µg) was analyzed for CNP transcripts using an exon 2-specific riboprobe. Probe and RNA were annealed at 50°C overnight and digested with RNase A and T1, and the resulting fragments were analyzed on an 8% denaturing polyacrylamide gel. Gels were fixed, dried under vacuum, and exposed to Kodak X-AR film at -80°C for 1 day. Results are representative of 3 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 4. Induction of uterine CNP gene expression by E2 in ovariectomized mice. A: mice were injected on the fifth day after ovariectomy with either vehicle (V, n = 2) or E2 (1 µg/20 g body wt). Some mice were injected with actinomycin D (n = 2; Act-D; 160 µg/20 g body wt) 2 h before E2 injection. Mice were killed 2 h after second injection, uterine horns were isolated, and total RNA was prepared. Total RNA (10 µg) was analyzed for CNP transcripts using an exon 2-specific riboprobe. Probe and RNA were annealed at 50°C overnight and digested with RNase A and T1, and the resulting fragments were analyzed on an 8% denaturing polyacrylamide gel. Gels were fixed, dried under vacuum, and exposed to Kodak X-AR film at 80°C for 1 day. Results are representative of 3 independent experiments. B: mice were injected on the fifth day after ovariectomy with either vehicle (V) or ICI-164,384 (ICI; 20 µg/20 g body wt) at 1000 h and vehicle (V) or E2 (1,000 ng/20 g body wt) at 1200 h as indicated. Mice were killed 2 h after the second injection, uterine horns were isolated, and total RNA was prepared. Total RNA (10 µg) was analyzed for CNP transcripts using an exon 2-specific riboprobe. Probe and RNA were annealed at 50°C overnight and digested with RNase A and T1, and the resulting fragments were analyzed on an 8% denaturing polyacrylamide gel. Gels were fixed, dried under vacuum, and exposed to Kodak X-AR film at -80°C for 1 day. Results are representative of 3 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

reasons: 1) plasma E2 followed closely the temporal pattern of uterine CNP expression; 2) E2 is known to increase after pregnant mare serum gonadotropin stimulation; and 3) the ovary is the predominant source of circulating E2. Accordingly, the ability of E2 to modulate uterine CNP expression was determined in ovariectomized mice. E2 significantly increased uterine irCNP in a dose-dependent fashion in ovariectomized mice (Fig. 1). A significant response was observed at a physiological dose (30 ng/20 g body wt), and maximum responses were observed with 100–1,000 ng/20 g body wt E2. Kinetically, the uterine irCNP response was rapid and maximal by 2 h after E2 injection (Fig. 2). This effect was specific for E2 because other steroids (testosterone, 2-methoxyestradiol, progesterone) failed to increase uterine irCNP at 6 h (Fig. 3). The weak response to estriol is consistent with its short-term pharmacokinetics (5).

Others have shown that CNP is a potent vasodilator, and the kinetics of CNP induction by E2 are consistent with the hypothesis that CNP mediates, in part, the effects of E2 on blood flow (24). E2 (10 ng/20 g body wt) administered to ovariectomized rats increases uterine blood flow after a brief delay of -1 h (32). Gradual increases in vascular permeability and edema occur during the next 5–8 h after E2 administration (5). These early uterine responses are followed during the next 24 h by endometrial and myometrial hypertrophy and hyperplasia. Local factors are thought to mediate E2-stimulated changes in uterine blood flow. Recent studies indicate that VEGF and nitric oxide may mediate some uterine vascular responses to E2. VEGF probably mediates a portion of the E2-dependent increase in uterine capillary permeability (5). However, VEGF is unlikely to mediate blood flow responses because it has relatively poor vasorelaxant activity. A role for nitric oxide in E2-stimulated uterine vasodilation was advanced from studies showing that N^G-nitro-L-arginine methyl ester could inhibit E2-stimulated blood flow. However, inhibition by N^G-nitro-L-arginine methyl ester was incomplete (only 60%), suggesting the participation of another vasodilator not affected by inhibition of nitric oxide synthase (31).

E2-stimulated increases in uterine CNP transcripts were blocked by prior administration of actinomycin D (Fig. 4A). This finding suggests that the changes in steady-state CNP mRNA effected by E2 are due to changes in de novo CNP transcription rather than by decreased rates of mRNA turnover. Injection of the specific nuclear ER antagonist, ICI-164,384, prevented subsequent activation of CNP gene expression by E2 (Fig. 4B). This result is consistent with the concept that the nuclear estrogen receptor is required for E2 to stimulate uterine CNP transcription. It is not clear whether in the uterus E2 acts on the Nppc gene through direct or indirect mechanisms. Cytokines such as TGF-β, interleukin 1, and tumor necrosis factor-α (TNF-α), enhance irCNP secretion from endothelial cells in vitro (25, 27, 28). These cytokines are synthesized in the mouse uterus (6), but the patterns of interleukin-1 and TNF-α expression are delayed relative to the CNP response that we observed; a role for TGF-β remains to be investigated.

Natriuretic peptide receptors (NPR1, NPR2) were previously demonstrated in random cycling rat uterus. Transcripts encoding NPR2 (that preferentially binds CNP) were expressed at a much greater level than NPR1 (8). It was not clear whether the receptors were regulated during the estrous cycle. An in situ binding study in random cycling rats showed ANP bound predominantly to the endometrium, with CNP bound to both the endometrium and myometrium (8). However, the CNP ligand ([125]Tyr0-CNP-22) used for this and similar binding studies recently has been questioned (30) because of oxidation of an intraring methionine residue that greatly reduced affinity for NPR2. Although it is clear that NPR2 is present in mammalian uterus, the cell specificity of expression and the potential for regulation by the hypothalamic-pituitary-gonadal axis remain to be addressed. A recent study suggested that in the rat ovary irCNP was increased at proestrus and that the NPR2 receptor transcripts in the rat ovary were modulated slightly during the estrous cycle (14). However, we have not observed significant variation in either CNP mRNA or irCNP in mouse (12) or rat ovary (unpublished observations). The signals regulating CNP expression in the ovary are not known and may well involve factors other than ovarian steroids.

The present study does not address the important question of which cell type(s) within the uterus can...
synthesize CNP. To date, in situ studies of CNP expression have focused on the central nervous system of adult male rats and mouse embryos (2, 15). Further work using in situ hybridization methods will be necessary to address this issue. Interestingly, when introduced into the central nervous system, E2 and CNP have similar effects on fluid homeostasis. E2 increases fluid intake through effects on hypothalamic regulatory centers that regulate pituitary hormone secretion. Intracerebral ventricular injection of CNP (0.1–10 pmol) stimulates fluid intake in ovariectomized rats deprived of water for 18 h (22). In addition, E2 and CNP have similar negative effects on gonadotropin (LH) secretion by anterior pituitary cells (16, 17, 21). Clearly, the results of the present study will be important for the design of experiments that address the biological function of CNP in reproductive organs, the vascular endothelium, and the central nervous system.

In conclusion, CNP, the most structurally conserved member of the natriuretic peptide family, is a potent vasodilator, smooth muscle relaxant, and inhibitory neuromodulator expressed by cells comprising the reproductive tract, by endothelial cells of the vasculature, and by neurons in the central and peripheral nervous systems. The present study shows that the mouse gene (Nppc) encoding CNP is regulated by estrogen at the transcriptional level in uterus. These results identify steroid hormones (estrogen and progesterone) as important physiological regulators of CNP expression in uterus. Further experiments will be necessary to elucidate the extent to which CNP mediates the biological effects of E2 in uterus and other sites of CNP biosynthesis, such as the central nervous system and vascular endothelium. Studies of the relationship between steroid hormones and CNP may eventually extend our understanding of mechanisms by which estrogen replacement therapy provides beneficial effects to the cardiovascular system of the postmenopausal female.

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