Heme Oxygenase in the Rat Anterior Pituitary: Immunohistochemical Localization and Possible Role in Gonadotropin and Prolactin Secretion

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The objectives of this study were to determine if heme oxygenase (HO), which catalyzes the degradation of heme and the formation of carbon monoxide (CO), is localized in the rat anterior pituitary and, if so, to determine if hemin (a substrate for HO) or chromium mesoporphyrin (CrMP) (an inhibitor of HO), alter pituitary gonadotropin and prolactin secretion. For localization of HO, sections of anterior pituitaries obtained from mature Holtzman Sprague-Dawley rats in different stages of the estrous cycle were immunostained for two of the HO isoforms, HO-1 and HO-2. The immunostaining for the inducible HO isoform (HO-1) was limited to discrete populations of pituitary cells, whereas the constitutive isoform (HO-2) had a more widespread distribution. The afternoon surge of leutinizing hormone (LH) in the plasma of ovariectomized, estradiol-treated rats was advanced by 2 hr after 7 days of treatment with CrMP (4 μ*M*/kg), and this effect was reversed when hemin (30 μ*M*/kg) was coadministered with CrMP. The afternoon follicle-stimulating hormone (FSH) surge was not affected by either treatment. In contrast, the afternoon prolactin (PRL) surge was completely blocked or delayed by CrMP treatment, and this effect was not reversed by hemin. *In vitro* perifusion of pituitary explants with CrMP also significantly reduced PRL release compared with secretion from untreated explants. *In vitro* gonadotropin-releasing hormone (GnRH)-stimulated FSH secretion was significantly increased from pituitary explants of ovariectomized, estradiol-treated rats treated *in vivo* with hemin but was unaffected by CrMP treatment, whereas GnRH-stimulated LH release was not affected by hemin but was increased by CrMP treatment. In conclusion, this study demonstrates that HO exists in the rat anterior pituitary gland, and that a substrate and an inhibitor of this enzyme alter the secretion of gonadotropins and PRL. Exp Biol Med 228:64–69, 2003

Key words: pituitary HO; gonadotropins; prolactin

The oxidative degradation of heme, which results in the formation of equimolar amounts of carbon monoxide (CO) and biliverdin, is catalyzed by the microsomal enzyme heme oxygenase (HO) (1). The localization and activity of this enzyme have been demonstrated in many organs and tissues, and its activity was reported to be highest in spleen, brain, and testes (2). In addition, three isoforms of HO have been identified and are referred to as HO-1, HO-2, and HO-3 (2, 3). HO-1, the inducible isoform, can be upregulated by heme, oxidative stress, metal ions, and other stimuli (3). HO-2, the constitutive isoform, has been shown to have a wide distribution in the body and a high concentration in the brain (4-6). HO-3, whose function is not yet known, is the newly identified isoform with weak heme catalytic activity (3). Immunohistochemical and *in situ* hybridization studies have revealed that the first two HO isoenzymes, HO-1 and HO-2, are both expressed in several areas of the rat brain (7). Various hypothalamic nuclei have been shown to have HO-1 and HO-2 mRNA proteins and enzymatic activity (7). It has also been demonstrated that HO-2 mRNA is expressed in the preoptic area (POA) and mediobasal hypothalamus (MBH) (8), and that the hypothalamus has a high CO production rate (9, 10).

The presence of HO in various structures of the nervous system or in structures involved with hormone secretion supports the role of CO, endogenously formed through the action of this enzyme, in neuronal transmission, neuroendocrine regulation, and other physiologic functions. CO appears to be implicated in the formation of hippocampal long-term potentiation and learning (11, 12). With regard to CO involvement in neuroendocrine regulation, recent experimental data show that CO modulates the release of corticotropin-releasing hormone (13, 14), arginine vasopressin (15,16), oxytocin (17), and gonadotropin-releasing hormone (GnRH) (18, 19) from the rat hypothalamus. Data on other possible effects of CO in modulating the function of the hypothalamus-pituitary-gonadal axis are unavailable. In this study, we attempted to localize HO in the rat pituitary gland and to determine whether providing a substrate or an inhibitor of HO has any effect on gonadotropin or prolactin

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(PRL) secretion, presumably by altering the levels of endogenously produced CO.

Material and Methods

Animals. The animals used in this study were Holtzman Sprague-Dawley rats purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed under controlled conditions of light (12:12-hr light:dark cycle), temperature (23°–26°C), and relative humidity (40%–55%) with free access to pelleted rat chow and tap water.

The investigations were approved by the University Animal Investigation Committee.

Materials. The hemin and chromium mesoporphyrin (CrMP) used in all experimental protocols were obtained from Porphyrin Products (Logan, UT) and were hydrated in 50 m*M* Na_2CO_3. α-Modified minimal essential medium (α-MEM), GnRH, dopamine, and thyrotropin-releasing hormone (TRH) were obtained from Sigma Chemical Company (St. Louis, MO). Polyestradiol phosphate (PEP, Estradurin) was obtained from Ayerst Laboratories (New York, NY).

Experimental Protocol. *Immunohistochemistry.* Holtzman Sprague-Dawley adult female rats in different stages of the estrous cycle were used in this study. They were euthanized by decapitation after CO_2 narcosis or anesthesia with ketamine and xylazine. Their pituitaries were collected, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), and embedded in paraffin. Ten-micrometer sections were deparaffinized for 10 min in xylene, and endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide in methanol for 15 min. A blocking solution (10% horse serum in PBS for HO-1 and 10% goat serum in PBS for HO-2) was then applied for 1 hr to prevent nonspecific protein binding. Sections were then incubated overnight in a humidified chamber at 4°C with a mouse monoclonal anti-HO-1 antibody (1:1000) or a rabbit polyclonal anti-HO-2 antibody (1:500; StressGen Biotechnology, Victoria, British Columbia, Canada) diluted in PBS. The next day, after rinsing in PBS, the sections were placed for 30 min in biotinylated secondary antibody reagent according to the Vectastain Elite Mouse IgG kit (Vector Laboratories, Burlingame, CA) protocol for HO-1 and Vectastain Elite Rabbit IgG kit (Vector Laboratories, Burlingame, CA) for HO-2, followed by a rinse in PBS. The sections were then incubated in the avidin-biotin reagent prepared according to the Vectastain ABC solution kit. After consecutive rinses in PBS, the sections were placed in diaminobenzidine solution for 5 min and were then cover slipped in PBS and glycerol. Omission of the primary antibody was included as a control and resulted in no immunostaining. Additional sections were stained with a Mallory stain for the pituitary gland (20). Photomicrographs were taken using a Leitz Laborlux optical microscope.

In Vivo Secretion of Leutinizing Hormone (LH), Follicle-Stimulating Hormone (FSH), and PRL. All rats used in these studies were bilaterally ovariectomized (OVX) and fitted with indwelling intraatrial Silastic catheters under ketamine (80 mg/kg) and xylazine (4 mg/kg) anesthesia. On the day of surgery, each rat was given 100 μg of polyestradiol phosphate in physiologic saline subcutaneously, and they were allowed to recover for 7 days before being randomly assigned to one of three treatment groups. After recovery, one group (the control) received daily subcutaneous injections of vehicle (50 m*M* Na_2CO_3) only; the second group received daily subcutaneous injections of CrMP (4 μ*M*/kg) for 7 days; and the third group received CrMP (4 μ*M*/kg) and hemin (30 μ*M*/kg) for 7 days. The doses for CrMP and hemin were selected based on the previous report of Vreman *et al.* (21) who measured CO in expired air of rats after treatment with these agents. At 0800–0900 on the day of the experiment, catheter extensions with an attached stopcock were added to the exteriorized portion of the indwelling catheter to facilitate blood sampling. Blood samples (0.3 ml) were collected every hour between 1200 and 1800 hr (5–11 hr after lights on). The samples were centrifuged after each sampling period and the red blood cells were resuspended in PBS and returned to the animal after the next sample. Plasma was collected by centrifugation and was frozen (–20°C) until subjected to radioimmunoassay (RIA). Hormone "surges" were defined within each animal as the sample with the maximal concentration of hormone. The time of occurrence of these surge samples from lights on in the animal room were noted, and this formed the reference point to which all other samples were aligned to obtain group means.

In Vitro GnRH-Stimulated LH and FSH Secretion. All rats used in these studies were OVX under ketamine (80 mg/kg) and xylazine (4 mg/kg) anesthesia and were given 100 μg of polyestradiol phosphate (PEP) by subcutaneous injection on the day of surgery. After a 7-day recovery period, rats were randomly assigned to the treatment groups. One group received daily subcutaneous injections with CrMP (4 μ*M*/kg) for 3 days, a second group received daily subcutaneous injections of hemin (30 μ*M*/kg) for 3 days, and a third group (control group) was given only vehicle (50 m*M* Na_2CO_3). On the day after the last injection, six rats from each treatment group were euthanized by CO_2 narcosis and decapitation. The anterior pituitaries were removed and the posterior pituitaries were discarded. The anterior lobes were placed in α–MEM containing 0.1% bovine serum albumin, and each was cut into four explants. Four randomly selected explants were placed in each 500-μl chamber of the perfusion apparatus (Acusyst S; Endotronics, St. Paul, MN). The chambers were perifused for 4 hr at 37°C at 100 μl/min with α–MEM gassed with 95% O_2 and CO_2. A 15-min GnRH pulse (500 ng) was given after the 1st hr of perifusion and another was given 1.5 hr later. The perfusate was collected at 10-min intervals directly after the initial placement of the pituitaries into chambers. After the 4-hr perifusion, the pituitary explants from each chamber were weighed and the collected culture medium fractions were frozen (–20°C) until subjected to RIA. The data re-

ported in the results section were obtained from six perifusion chambers each for vehicle-, CrMP-, and hemin-treated group.

In Vitro PRL Secretion. For these studies, only animals in proestrous phase of the estrous cycle were used. On the day of the experiment, six rats were euthanized by CO_2 narcosis and decapitation. The anterior pituitaries were removed and the posterior pituitaries were discarded. The anterior lobes were placed in α–MEM containing 0.1% bovine serum albumin and 0.1% ascorbic acid, and each was cut into four explants. Two randomly selected explants were placed in each 500-μl chamber of the perfusion apparatus described above. The chambers were perifused for 3 hr at 100 μl/min with α-MEM warmed to 37°C and gassed with 95% O_2 and 5% CO_2. The perifusion medium delivered to six chambers in each experiment contained CrMP (4 μ*M*), whereas medium to six other chambers did not contain CrMP. During the 1st hr, the perifusion medium to all 12 chambers contained dopamine (DA; 1 μ*M*), but during a 2nd hr, DA was not present in the perifusion medium. During the 3rd hr, DA was again added to the medium at 1 μ*M*, and, for the last 30 min of the perifusion, TRH (10 n*M*) was also added. The perfusate from each chamber was collected at 10-min intervals directly after the initial placement of the pituitaries into chambers.

At the conclusion of the experiment, the pituitary explants in each chamber were weighed, and the collected medium fractions were frozen (–20°C) until subjected to RIA. The data reported in the results section were combined from two separate perifusion experiments.

RIA. LH, FSH, and PRL were measured in the culture media or plasma samples using double antibody RIAs with reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). All samples and standards were assayed in duplicate, and the standards used were rLH-RP-3, rFSH-RP-2, and rPRL-RP-3. The data were reported as picograms or nanograms per milligram anterior pituitary.

Statistical Analysis. The data were analyzed using analysis of variance (ANOVA) and the differences between the treatment groups were evaluated by Tukey's or Dunnett's *post hoc* tests. Repeated measures ANOVA, the preferred method, could not be used because of missing samples in both the *in vivo* and *in vitro* studies. Data were expressed as means ± SEM. Means that were different at the $P < 0.05$ level were considered to be significant.

Results

Immunohistochemistry. Immunohistochemical localization of HO in the anterior pituitary is shown in Figure 1. HO-1 immunostaining (Fig. 1A) appeared to be limited to discrete population of cells, whereas HO-2 immunostaining (Fig. 1B) showed a more widespread distribution. The omission of the primary antibody in the HO-1 protocol (control for HO-1) resulted in no staining, as shown Figure 1C. The omission of the primary antibody for HO-2 showed a similar pattern (data not shown). No attempt was made to specifically identify which types of pituitary cells stained positively for HO-1 and HO-2, but Figure 1D shows a Mallory-stained section with lightly staining chromophobic cells (likely corticotrophs), basophilic cells (probably thyrotrophs or gonadotrophs), and acidophils (most likely lactotrophs or somatotrophs).

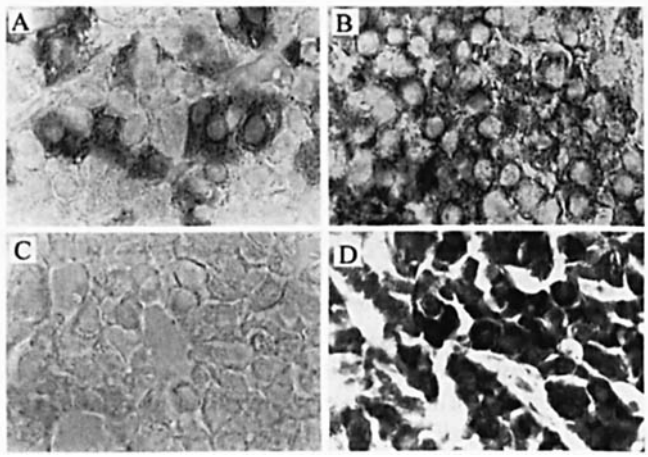

Figure 1. Immunohistochemical localization of HO in the anterior pituitary of mature Holtzman Sprague-Dawley female rats. (A) HO-1. (B) HO-2. (C) Control for HO-1. (D) Mallory stain. Photomicrographs were taken at 500× magnification.

***In Vivo* LH, FSH, and PRL Secretion.** The effects of *in vivo* treatments with CrMP or CrMP and hemin on the estradiol-induced afternoon surges of LH, FSH, and PRL in plasma are shown in Figure 2. Figure 2A shows that vehicle-treated control rats exhibited a small but significant LH surge at 8 hr after lights on (1500 hr clock time), whereas CrMP (4 μ*M*/kg) administered alone for 7 days advanced the LH surge approximately 2 hr. Administration of hemin (30 μ*M*/kg) concurrently with CrMP returned the LH surge to the time observed in vehicle-treated rats (~8 hr after lights on), but also increased LH secretion at other times during the afternoon.

The amplitude or timing of the afternoon FSH surge was not significantly altered by CrMP or CrMP + hemin treatments compared with vehicle-treated rats (see Fig. 4B).

An afternoon PRL surge occurred approximately 10 hr after lights on (1700 hr clock time) in vehicle-treated rats (see Fig. 2C). Treatment with CrMP either completely blocked or delayed the PRL surge and this effect of CrMP was not altered by concurrent treatment with hemin.

***In Vitro* GnRH-Stimulated LH and FSH Secretion.** GnRH-stimulated release of LH and FSH, summed over the 4-hr perifusion period, is shown in Figure 3. LH release from pituitary explants obtained from OVX, estradiol-primed rats treated with hemin (30 μ*M*/kg) was not different from that observed from pituitaries of vehicle-treated rats (Fig. 3A). In contrast, CrMP treatment significantly increased LH release. GnRH-stimulated FSH release was increased by hemin, whereas CrMP did not have any significant effect (Fig. 3B).

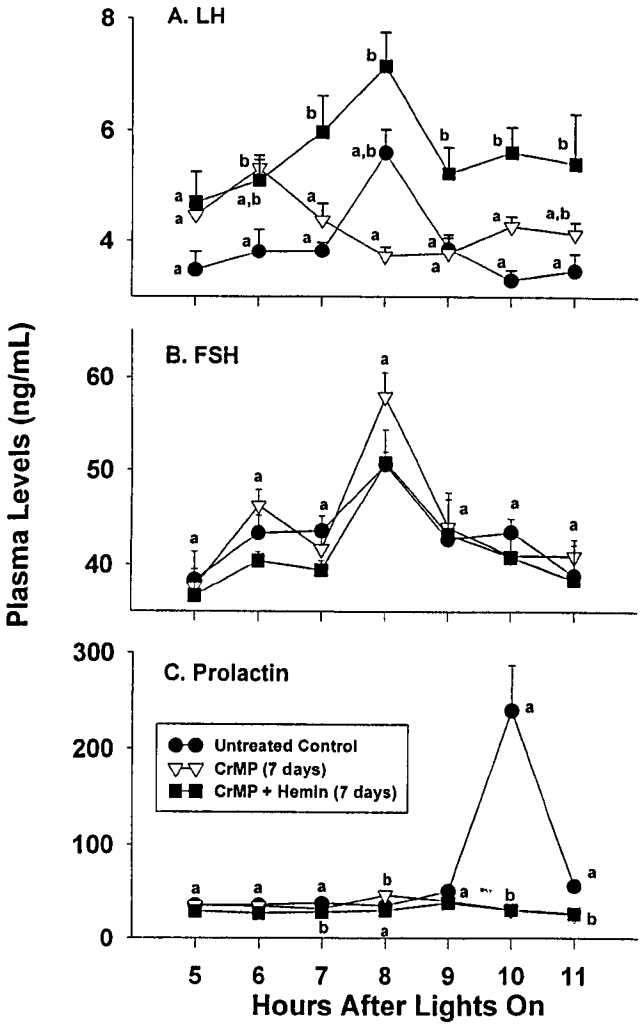

Figure 2. The effects of *in vivo* treatment with CrMP alone or combined with hemin on the afternoon surges of LH (A), FSH (B), and PRL(C) in ovariectomized, polyestradiol phosphate-treated Holtzman Sprague-Dawley rats. [a, b] denote means (±SEM) across groups at the same time interval that have different letters are statistically different ($P < 0.05$; $n = 7$–11).

***In Vitro* PRL Secretion.** PRL release from the pituitary explants, which were obtained from proestrous rats and treated with CrMP *in vitro,* was significantly suppressed by exposure to 1 μ*M* dopamine (30- to 60-min period vs the 0- to 30-min period; $P < 0.05$), whereas there was no significant reduction in PRL secretion from similar explants not subjected to the *in vitro* CrMP treatment (Fig. 4A). Withdrawal of DA from the perifusion medium induced an increase in PRL release in both groups, but the response was observed earlier in the CrMP-treated pituitary explants (90- to 120-min period) than in untreated explants (150- to 180-min period). To directly compare CrMP and control groups, data were transformed to a percentage of the level in the initial 30-min perifusion period (Fig. 4B). CrMP treatment significantly suppressed PRL release during the first 30-min period when DA was present and in the last 30 min when both DA and TRH were present when compared with the respective values in the control group.

Discussion

Our study demonstrates that HO-1 and HO-2 isoenzymes are localized in the pituitary gland of the female rat. The staining observed for HO-1 was confined only to small population of cells, whereas the immunostaining for HO-2 was more widespread, suggesting that, in the pituitary gland, the levels of HO-1 are low and its expression is limited to certain types of cells, whereas HO-2 appears to be more highly expressed. This hypothesis is in good agreement with what has been previously observed about the presence of HO isoenzymes in other tissues, such as the brain. It has been shown that HO-1 mRNA and protein are generally found in very low concentrations in the brain until induced by specific stimuli (such as following heat shock or cerebral hypoxia) (5, 6, 22). In contrast, HO-2, the constitutive isoform, has been found to be widely expressed in brain and several hypothalamic nuclei such as preoptic area, mediobasal hypothalamus, and paraventricular and supraoptic nuclei (3, 5, 8, 23, 24), areas important in the regulation of the anterior pituitary secretion. The morphologic data in the current study show that anterior pituitary cells contain the enzyme necessary for the production of endogenous CO and this suggests that CO may have an intracrine,

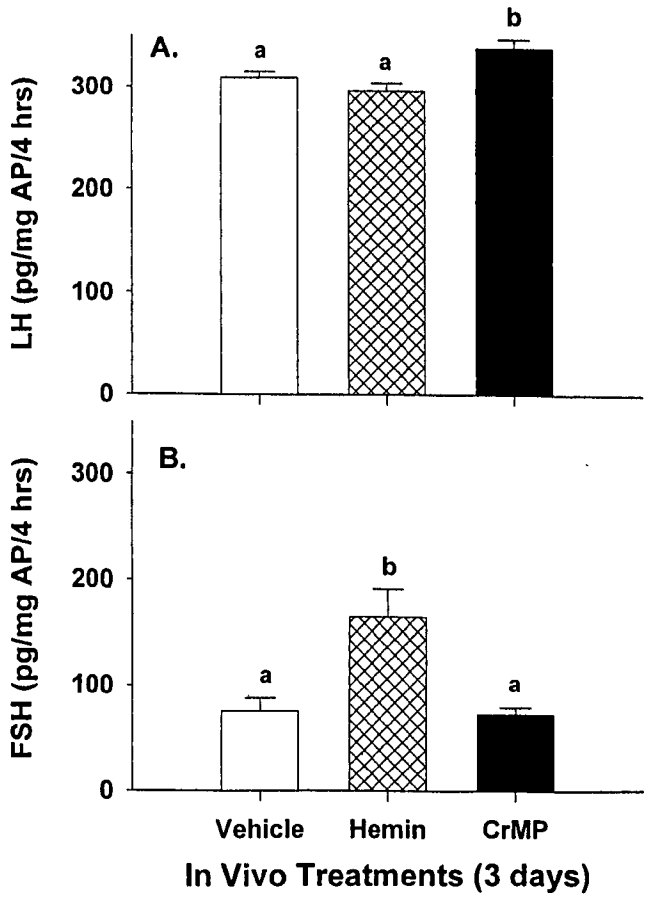

Figure 3. The effects of *in vivo* treatment with hemin or CrMP on *in vitro* GnRH-stimulated LH (A) and FSH (B) release from pituitaries of ovariectomized estrogen-treated rats. [a, b] denote means (±SEM) that have different letters are statistically different ($P < 0.05$; $n = 6$).

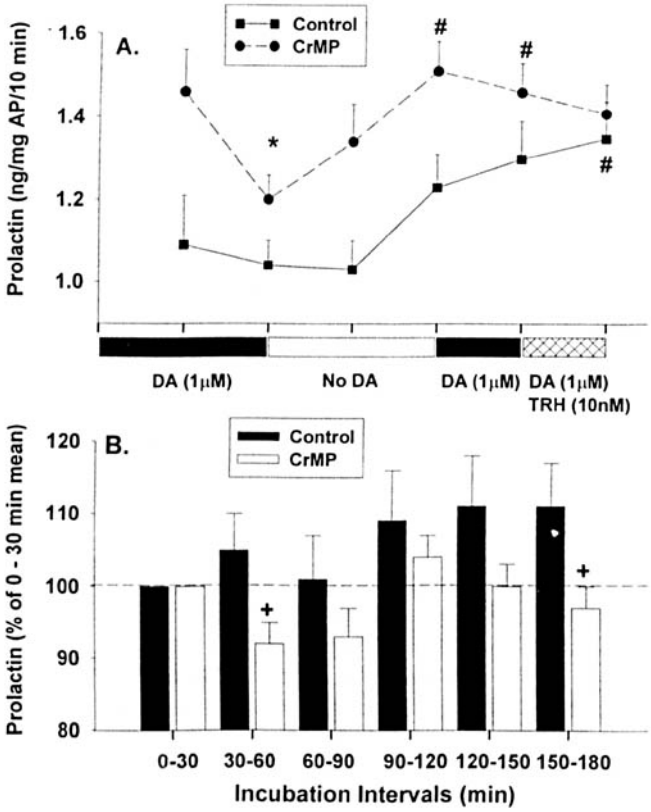

Figure 4. The effects of *in vitro* treatment with CrMP on prolactin release in the presence or absence of DA and TRH. (A) Absolute levels of prolactin. * Significantly different from 0- to 30-min value ($P < 0.05$); # Significantly different from 30- to 60-min value ($P < 0.05$). (B) Data transformed to percentage of 0- to 30-min mean for each perifusion chamber. + CrMP vs. control within incubation intervals ($P < 0.05$).

autocrine, or paracrine role in the regulation of pituitary hormone secretion. Although we did not specifically identify the types of pituitary cells that showed HO immunostaining, this may not be necessary because CO, like nitric oxide, is a very soluble gas capable of diffusing rapidly through cell membranes and gaining access to neighboring cells. This argument notwithstanding, future studies are needed to determine which pituitary cells contain HO because this enzyme may have other actions besides the production of CO.

In our studies, hormone release from the anterior pituitary appears to be influenced by agents that would alter endogenous levels of CO. That the estradiol-induced afternoon surge of plasma LH was advanced by chronic administration of an HO inhibitor (CrMP) and that the concurrent administration of a substrate of HO (hemin) could reverse this timing effect as well as increase overall levels of LH at other times in the afternoon (Fig. 2) suggests that CO may be a positive modulator of *in vivo* LH secretion. However, that CrMP administration enhanced GnRH-induced LH secretion the pituitary explants *in vitro* (Fig. 3A) indicates that the positive modulation must be greatest in the hypothalamus. It has been have shown that *in vitro* GnRH release is stimulated by the HO substrate hematin and this effect is reversed by an HO inhibitor (zinc protoporphryin) (20). However, it has also been shown that hemin infusion into the medial preoptic area and median eminence-arcuate nucleus complex where the GnRH neurons are located did not affect the timing of gonadotropin surges (19), and that the levels of mRNA for HO-2 in the hypothalamus do not change during the LH surge on proestrus (25). Clearly, additional studies are needed to clarify the relationship between hypothalamic and pituitary levels of CO and the regulation of LH secretion.

Our observation that estradiol-induced FSH secretion was not altered by either CrMP or CrMP + hemin treatment (Fig. 2A) suggests that CO may not have a significant role in the overall regulation of FSH secretion *in vivo*. However, that hemin treatment enhanced, but CrMP did not reduce, FSH secretion by pituitary explants *in vitro* suggests some potential positive modulation by elevated, but not basal, levels of CO on FSH release at the level of the pituitary.

A positive modulation by CO on PRL secretion is also suggested by our results that the estradiol-induced afternoon PRL surge was either blocked or delayed by CrMP treatment (Fig. 2C) and that DA-induced inhibition of PRL release from pituitary explants was significantly enhanced by acute CrMP exposure *in vitro*. These results suggest that, similar to nitric oxide, CO appears to have a stimulatory effect on PRL secretion.

Taken together, the results of our studies suggest that CO could be produced in the anterior pituitary gland by the action of HO and that providing HO with substrate or blocking its action with an inhibitor influences the secretion of gonadotropins and PRL.

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