Heme Oxygenase in the Rat Ovary: Immunohistochemical Localization and Possible Role in Steroidogenesis

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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 ovary and, if so, to determine if hemin (a substrate for HO) or chromium mesoporphyrin (CrMP, an inhibitor of HO), alter basal or gonadotropin-induced steroidogenesis. The hypothesis was that CO produced endogenously by HO suppresses steroid hormone production by the ovary similar to the action of nitric oxide. For the histological localization of HO, sections of ovaries obtained from mature Holtzman Sprague-Dawley rats were immunostained for two of the HO isoforms, HO-1 and HO-2. Theca cells and granulosa cells of follicles and luteal cells stained for HO-1, whereas the ovarian stroma showed a low intensity of staining. Theca, granulosa cells, and corpora lutea as well as the ovarian stroma exhibited HO-2 staining. HO-2 immunostaining appeared more intense for theca cells than granulosa cells. In the study of steroidogenesis, three daily injections of hemin stimulated basal- and gonadotropin-induced androstenedione and estradiol secretion from ovaries of pregnant mare serum gonadotropin-treated immature rats *in vitro*, but had no effect on progesterone production. A similar treatment with CrMP suppressed basal- and gonadotropin-induced secretion of progesterone and androstenedione, but had no effect on estradiol production. These data, taken together, show the existence of HO in the rat ovary and suggest a possible stimulatory role of endogenous CO in the production of ovarian steroids. Exp Biol Med 228:59–63, 2003

Key words: ovarian HO; steroid production

It has long been known that carbon monoxide (CO) is synthesized endogenously during the catabolism of heme through the action of heme oxygenase (HO) (1–3), but CO has only recently emerged as another endogenously produced gas with regulatory functions, similar to nitric oxide (NO).

Although less studied than NO, CO has been shown to share with NO several biological functions such as platelet activation (4), vasodilation (5), and maintenance of uterine quiescence during pregnancy (6, 7). As a neurotransmitter, CO has been shown to influence hypothalamic secretion of corticotropin-releasing hormone (8), vasopressin (9), and oxytocin (10). CO stimulates *in vitro* GnRH secretion (11), whereas *in vivo* GnRH release appears unaffected by CO (12).

That CO has diverse biological functions is supported by the fact that HO, the enzyme responsible for most of the endogenous production of CO, has been detected in many tissues (3, 13). Three isoenzymes, HO-1, HO-2, and HO-3, have been reported (13–16). The inducible isoform, HO-1, is upregulated by heme, oxidative stress, metal ions, and hormones (14, 17), whereas HO-2, detected in brain (18), gastrointestinal tract (19–21), spleen, and liver (22) is a constitutive isoform. HO-3, a newly identified isoform, has weak heme catalytic activity, but appears to play a regulatory role in heme-dependent processes (13).

Both HO-1 and HO-2 isoforms have been identified in reproductive organs such as testes (15), human and guinea pig placenta (23–25), and in pregnant and nonpregnant uterus (7, 26). To date, information pertaining to the existence of HO in the ovaries is limited to the observation that HO activity can be induced by metal ions (27). Because HO has been shown to degrade cytochrome P-450 enzymes (3, 28, 29) and because several enzymes in the steroid synthetic pathway are heme-containing P-450 proteins, HO could affect ovarian steroidogenesis by regulating these enzymes. That NO, like CO, binds to heme groups and inhibits ovarian steroidogenesis (30) suggests that CO may have a similar inhibitory action on steroid production. Therefore, the purpose of the present study was to attempt to localize HO in the rat ovary by immunohistochemistry and to determine the possible effects of manipulating the activity of HO, using a substrate or an inhibitor, on progesterone, androstenedione, and estradiol production.

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Received September 10, 2001.
Accepted August 15, 2002.

1535-3702/03/2281-0059$15.00

Material and Methods

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 ketamine and xylazine anesthesia. Their ovaries 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 was then applied for 1 hr to prevent nonspecific protein binding. The blocking solutions were 10% horse serum in PBS for HO-1 and 10% goat serum in PBS for HO-2. Sections were then incubated with monoclonal mouse anti-HO-1 antibody (1:1000) or polyclonal rabbit anti-HO-2 antibody (1:500; StressGen Biotechnology. Victoria, British Columbia, Canada) diluted in 0.1 *M* PBS overnight in a humidified chamber at 4°C. The next day, the sections were rinsed in 0.1 *M* PBS and were placed in biotinylated secondary antibody reagent according to the protocols provided with the Vectastain Elite Mouse IgG kit for HO-1 and Vectastain Elite Rabbit IgG kit for HO-2 (Vector Laboratories, Burlingame, CA), followed by a rinse in 0.1 *M* PBS. The sections were then incubated for 30 min in the avidin-biotin reagent prepared according to the protocol in the Vectastain ABC solution kit. After consecutive rinses in PBS, the sections were placed in diaminobenzidine solution for 5 min were and then cover slipped in PBS and glycerol. Omission of the primary antibody was included as a control and resulted in no immunostaining. Photomicrographs were taken after the immunostaining using a Leitz Laborlux optical microscope.

Animals. The animals used in this study were immature Holtzman Sprague-Dawley female rats, purchased from Harlan Sprague Dawley (Indianapolis, IN), and were group housed under controlled conditions of light (lights on 0700–1900 hr), temperature (70°–72°F), and relative humidity (40%–55%) with free access to pelleted rat chow and tap water.

The procedures were approved by the University Animal Investigation Committee.

Materials. The hemin and metalloporphyrins used in all experimental protocols were obtained from Porphyrin Products (Logan, UT) and were hydrated in 50 m*M* Na_2CO_3. Culture medium (α-modified minimal essential medium; α-MEM), pregnant mare serum gonadotropin (PMSG), and human chorionic gonadotropin (hCG) were obtained from Sigma Chemical Company (St. Louis, MO).

Experimental Protocol. Groups of six 23-day-old Holtzman Sprague-Dawley female rats each received three daily subcutaneous injections of PMSG (25 IU/rat) at 0900–1000 hr to induce ovarian development and function. Some groups were also treated with hemin (30 μ*M*/kg; s.c.), or chromium mesoporphyrin (CrMP; 4 μ*M*/kg; s.c.) concurrently with PMSG. The doses of hemin and the CrMP were selected based on data in which CO levels were measured in rats before and after the administration of these agents (31). HO activity and CO levels were not directly measured in this study.

On the experimental day, the rats were euthanized and the ovaries were collected, trimmed of fat, oviducts, and ovarian bursa, and each ovary was cut into fragments. The ovarian fragments were placed into wells of 24-well tissue culture plates containing α–MEM or α–MEM containing PMSG (1 IU/ml) with hCG (1 IU/ml). The plates were incubated for 4 hr in a humidified atmosphere of 50% O_2 and 5% CO_2 at 37°C. Every hour, the ovarian fragments were moved to wells with fresh medium of the same composition. At the conclusion of incubation, the medium was collected from each well and the ovarian fragments were weighed. The collected medium samples were pooled across the 4-hr incubation period for each ovarian fragment, and the pooled samples were assayed for progesterone, androstenedione, and estradiol using commercial solid phase radioimmunoassay kits (Coat-a-Count; Diagnostic Product Corporation, Los Angeles, CA). All samples and standards were assayed in duplicate. The hormonal concentration of the medium was expressed per milligram of ovary.

Statistical Analysis. The *in vitro* steroid production data were pooled across three to six experiments and were subjected to two-factor analysis of variance (ANOVA; factor 1: the 3 *in vivo* treatments, and factor 2: the 2 *in vivo* treatments). After the determination that there were significant effects of one or both types of treatments, specific differences between *in vitro* treatments within each *in vivo* treatment were determined by F-test, whereas specific differences between *in vivo* treatment groups and the control within each *in vitro* treatment were tested using one-factor ANOVA and Dunnett's *post hoc* test (SigmaStat; SPSS, Chicago, IL). A value of $P < 0.05$ was regarded as significant.

Results

Immunohistochemistry. HO-1 and HO-2 immunoreactivity was observed in all the ovaries studied and representative photomicrographs are presented in Figures 1 and 2. HO-1 was present in the corpus luteum (CL), whereas staining in the ovarian stroma (S) was absent or of very low intensity (Fig. 1A). HO-1 immunostaining was also observed in theca cells (TC; Fig. 1B), as well as in the granulosa cells (GC; Fig. 1C). Omission of the primary antibody for HO-1 resulted in no staining (Fig. 1D). HO-2 appears to be present in each ovarian structure (Fig. 2). Figure 2A shows HO-2 immunostaining in the CL as well as in the S. TC and GC also exhibited HO-2 staining, with TC having a higher staining intensity than the GC (Fig. 2B). The control section for HO-2 is shown in Figure 2C.

Ovarian Steroidogenesis Studies. Figure 3 shows the effects of three daily injections of the HO inhibitor CrMP and the HO substrate hemin on *in vitro* ovarian

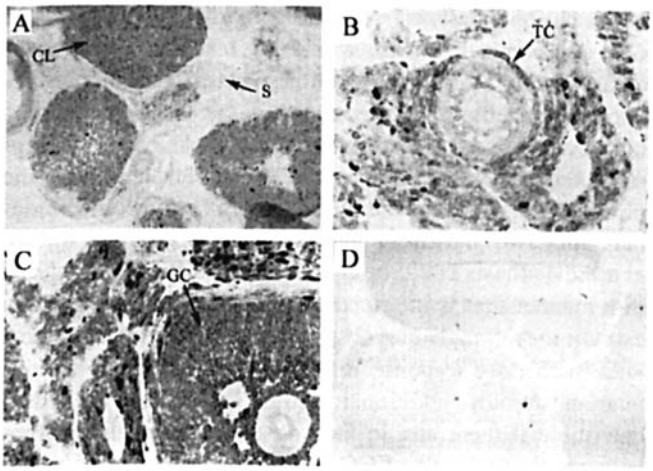

Figure 1. Immunohistochemical localization of HO-1 in the ovary of mature Holtzman Sprague-Dawley female rats. (A) Localization of HO-1 in S and CL (×2.5 objective). (B) Localization of HO-1 in TC (×10 objective). (C) Localization of HO-1 in GC (×10 objective). (D) Control for HO-1 (×2.5 objective).

progesterone, androstenedione, and estradiol secretion. Figure 3A also shows that CrMP significantly reduced whereas hemin had no effects on basal progesterone secretion compared with the control group. Gonadotropin-induced progesterone production was not affected by CrMP or hemin treatment. CrMP treatment had no effect on basal but significantly reduced gonadotropin-induced androstenedione secretion, whereas the 3-day treatment with hemin significantly stimulated basal but not gonadotropin-induced androstenedione production (Fig. 3B). Estradiol production was not influenced by CrMP treatment, but hemin significantly increased gonadotropin-induced secretion while having no effect on basal estradiol production (Fig. 3C).

Discussion

HO activity has been detected in many organs and tissues, including those producing steroids (14). To date, the only information available on the ovarian HO is that its activity could be induced by metal ions (27), suggesting it is HO-1, the inducible isoform. Because content and activity of cytochrome P-450 enzymes have been shown to be decreased by HO (28, 29) and a number of catalytic steps in the biosynthesis of ovarian steroids are mediated by heme-containing cytochrome P-450 enzymes, it seems probable that HO could regulate ovarian steroidogenesis. In addition, an interdependent relationship has been shown to exist between the pathways of steroidogenesis and heme metabolism where changes in the heme biosynthesis and degradation are reflected in the cytochrome P-450 concentration (32–34).

The present study demonstrates the localization in the rat ovary of not only the HO-1 isoform, which agrees with the activity data of others (27), but also the presence of HO-2, the constitutive isoform of HO. That HO was observed in all or nearly all of the structures of the ovary (follicular TC and GC, and CL for HO-1 and TC, GC, CL,

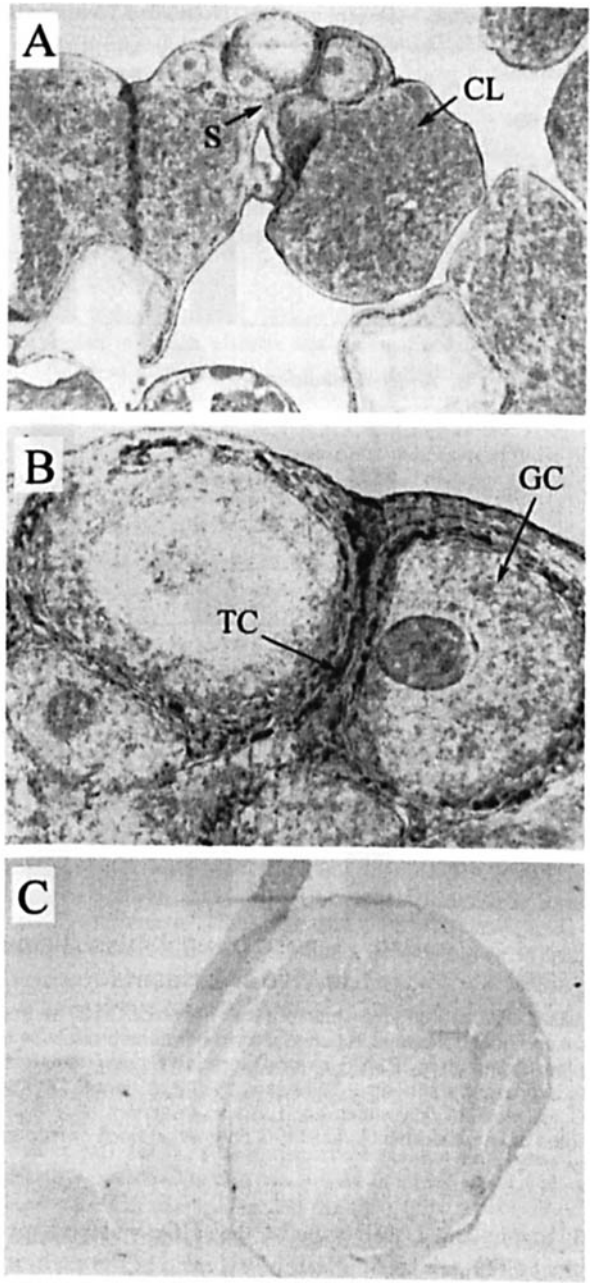

Figure 2. Immunohistochemical localization of HO-2 in the ovary of mature Holtzman Sprague-Dawley female rats. (A) Localization of HO-2 in S and CL (×2.5 objective). (B) Localization of HO-2 in TC and GC (×10 objective). (C) Control for HO-2 (×2.5 objective).

and S for HO-2) suggests that this enzyme or its products could have wide ranging effects on ovarian function. HO localization has been demonstrated by other groups in reproductive tissues such as testes (15, 17, 27), pregnant and nonpregnant uterus (7, 26), placenta (24, 25), as well as in nonreproductive tissues such as spleen, liver (14, 22), gastrointestinal tract (19–21), and brain (18). In the pregnant myometrium, HO-1 has been shown to be upregulated by progesterone (7). Upregulation by progesterone could explain the intense HO-1 immunostaining in the CL in the current study.

Our observations on the effects of chronic treatment

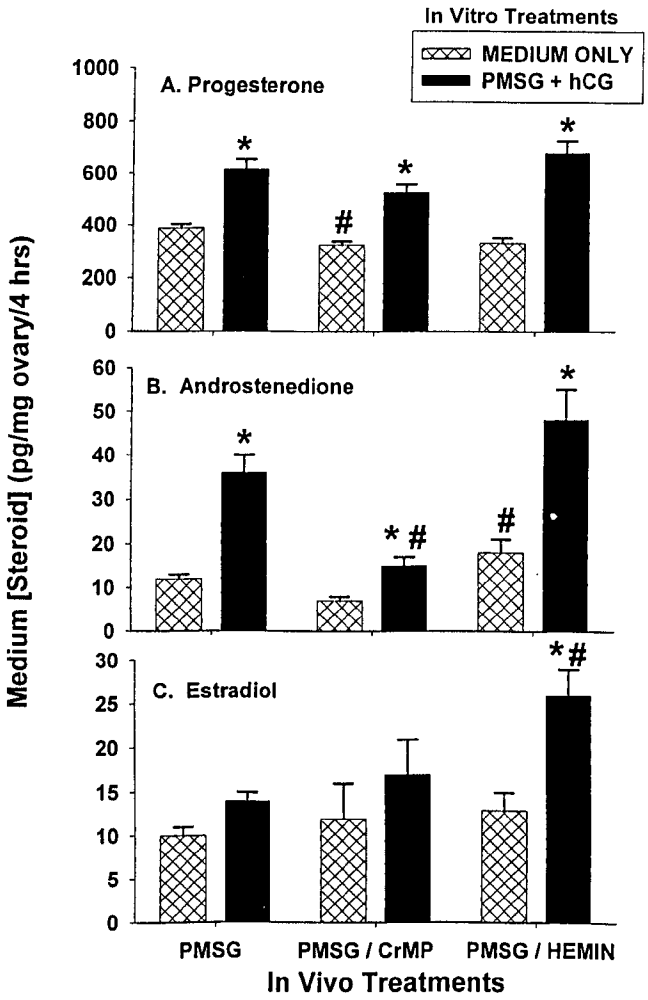

Figure 3. Effect of *in vivo* treatment with hemin or CrMP on *in vitro* basal and gonadotropin-stimulated steroid hormone production from ovaries of immature, PMSG-treated Sprague-Dawley female rats. Data expressed as mean ± SEM (n = 15–33). * denotes PMSG + hCG vs. medium only within each *in vivo* treatment ($P < 0.05$). # denotes *in vivo* treatments vs. PMSG only within each *in vitro* treatment ($P < 0.05$).

with hemin and CrMP suggest that HO, and perhaps its product CO, may be implicated in steroid hormone biosynthesis by the ovary. That hemin treatment stimulated (presumably by elevating endogenous levels of CO) while CrMP treatment inhibited steroid production (presumably by reducing endogenous CO levels) suggests that endogenous CO may be a positive modulator of steroidogenesis in contrast to NO, which has been shown to reduce steroid production from the rat ovary (29).

That hemin increased androstenedione and estradiol but not progesterone production, whereas CrMP reduced progesterone and androstenedione but not estradiol secretion suggests the possible roles of HO and its products like CO are likely to be complex. On possible explanation for our current results is that CO binds to the heme-containing steroidogenic enzymes and stimulates ovarian steroid production in dose-response relationships that are different for the various steroidogenic pathways. Progesterone synthesis could be maximally upregulated at normal tissue levels of CO, and hemin treatment would not result in a further increase in progesterone production, whereas CrMP could reduce progesterone by decreasing tissue CO below the normal level. In contrast, estradiol synthesis may only be upregulated when tissue levels of CO are elevated above the normal level. If such were the case, then hemin could stimulate, but CrMP may not inhibit estrogen production. Androgen biosynthesis could be regulated by tissue levels of CO in a manner that is intermediate between progesterone and estradiol. Although our observations would be consistent with such dose-response differences between the various ovarian steroids, additional studies are clearly needed to determine if there are, in fact, dose-response relationships between tissue levels of CO and production of ovarian steroids and, if so, whether such relationships operate differently for the various steroidogenic pathways.

A second possibility is that there is some type of functional interaction between HO and the P-450 steroidogenic enzymes that does not involve CO or other products of HO-catalyzed degradation of heme. Some earlier reports suggested that HO can directly degrade P450 heme-containing proteins (3, 28, 29), whereas others have suggested that HO could deplete the cellular pool of heme necessary for the maintenance of P450 enzyme synthesis (35). If induction of HO activity by hemin caused a reduction in P450 steroidogenic enzymes, then one might expect reduced ovarian steroid production after hemin treatment. This result was not the outcome of our current studies. However, that HO inhibitors can also induce HO (34), which could then decrease levels of P450 steroidogenic enzymes, could explain the inhibitory effects of CrMP we observed.

In summary, the results of our studies show that both HO isoenzymes are present in the rat ovary with HO-1 detected in TC, GC, and CL; and HO-2 found in the TC, GC, CL, and ovarian S; *in vivo* treatment with hemin, a substrate for HO, stimulates androstenedione and estradiol secretion; and *in vivo* treatment with CrMP, an inhibitor of HO, decreases progesterone and androstenedione secretion.

We conclude that CO may be produced endogenously in the rat ovary by ovarian HO and that this endogenous CO may play a stimulatory role in ovarian steroid hormone biosynthesis. Confirmation of these conclusions awaits direct measurement of CO production by the ovary and the observation that CO directly alters activity of steroidogenic enzymes.

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