The Expression of a Mitochondria-Localized Glutamic Acid-Rich Protein (MGARP/OSAP) Is Under the Regulation of the HPG Axis

Mingxue Zhou,* Yifeng Wang,* Shaoling Qi, Jian Wang, and Shuping Zhang

State Key Laboratory of Biomembrane and Membrane Biotechnology, School of Life Sciences, Tsinghua University, Beijing 100084, China

The hypothalamic-pituitary-gonadal (HPG) axis exerts a profound effect on animal development, reproduction, and response to stress, and new insights into its complicated functional activities are continuously being made. In the present study, by using immunohistochemical studies and different mouse models (ovariectomy and ob/ob mice), we systematically analyzed the expression of a novel mitochondria-localized glutamic acid-rich protein (MGARP)/ovary-specific acid protein and demonstrated that MGARP is under the regulation of the HPG axis. MGARP is highly enriched in steroidogenic tissues and the visual system. Interestingly, its expression increases as mice develop. Early in development, MGARP is mainly detected in the retina and adrenal gland. At this early developmental stage, its expression is not detectable in the gonads, but its expression in the gonads dramatically increases during the first 2–4 wk after birth. Importantly, MGARP levels correlate with estrogen levels in the ovaries during the estrous cycle, and estrogen regulates the expression of MGARP in a tissue-specific manner and through a feedback regulatory mechanism. Functional inhibition of GnRH with an antagonist strongly reduces MGARP levels, and knockout of leptin (ob/ob) significantly reduces the MGARP expression in follicular granular cells. We proposed a model that elucidates the role MGARP plays in the HPG axis. Within the HPG axis loop, MGARP participates in hormone biosynthesis while being under the regulation of the hormones derived from the HPG axis. (*Endocrinology* 152: 2311–2320, 2011)

Steroid hormones have a variety of metabolic activities, including neuromodulatory, neuroendocrine, and neuroprotective effects, and they play crucial roles in mammalian reproduction, development, aging, and stress responses by acting on the brain and other organs (1, 2). All of these activities of steroids are critically regulated by the hypothalamic-pituitary-gonadal (HPG) axis (1, 3, 4). In the HPG axis, GnRH, a factor secreted by the hypothalamus, travels down the anterior portion of the pituitary via the hypophyseal portal system and binds to the receptors on the secretary cells of the adenohypophysis (5). Pulsatile release of GnRH stimulates the secretion of LH and FSH in the adenohypophysis (6). LH regulates the synthesis of the gonadal hormones and ovulation, whereas FSH promotes ovarian follicle maturation, estrogen release, and spermiogenesis. Both LH and FSH participate in the regulation of the estrous cycle, menstrual cycle, gonad development, and reproduction. Furthermore, androgen and estrogen use a feedback mechanism to regulate GnRH, LH, and FSH biosynthesis (7). The HPG axis is an essential and very complicated system throughout life, and there are still many questions remaining to be answered.

The mitochondria-localized glutamic acid-rich protein (MGARP) is a novel mitochondrial protein identified by large-scale screenings for genes specifically expressed in the ovary, retina, cornea, and adrenal gland (8–13). It was previously named as mouse ovary-specific acidic protein and human corneal endothelium-specific protein (8, 13), but its function was not substantially defined. In our previous report, we proposed...
an accordant and universal name, MGARP, for the protein in consideration of its enrichment with glutamic acids and specific cellular localization (11). Our study on MGARP in the retina showed robust levels of expression in the inner segment of the photoreceptor, outer plexiform layer, and ganglion cell layer of the retina (11). A reduction in MGARP expression results in mitochondrial fragmentation and overexpression of MGARP with a deletion of the N terminus causes severe mitochondrial aggregation (10, 11). In Y-1 cells, knockdown of MGARP significantly inhibits 8-bromoadenosine-cAMP-induced progesterone production (10). The detailed regulatory mechanisms controlling MGARP expression, however, still remain to be elucidated. It is also unclear how MGARP interacts with the different elements of the HPG axis, which includes the main components of both the neurosecretory and steroidogenic systems.

In this study, we systemically analyzed the expression and the regulatory network of MGARP by Western blotting and immunohistochemical analysis using different mouse models and found that MGARP is predominantly expressed in the steroidogenic tissues and the compartments of the visual system. We also found that MGARP expression increases as the gonads develop. It can be regulated by estrogen, GnRH, and leptin, all of which are regulators or effectors of the HPG axis. Our results suggest that the interaction between MGARP and elements of the HPG axis forms a functional loop, with steroid hormones as the mediators.

Materials and Methods

Reagents and animals

17β-Estradiol (E2) (E8875) and leptin (L3772) were purchased from Sigma (St. Louis, MO). Cetrorelix acetate was from Shanghai Taishi Biotecnology Co., Ltd. (Shanghai, China). ICR mice were bred by the Animal Facility of Tsinghua University. Ob/ob mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed in compliance with the relevant laws and institutional guidelines. The animal care procedures were institutionally reviewed by the Institutional Animal Care and Use Committee of Tsinghua University. The euthanasia was performed by adopting 1% sodium pentobarbital by ip injection.

Antibody preparation

The full-length MGARP cDNA sequence was inserted into pGEX-4T-1 vector and transformed into the BL21 expression strain. Isopropyl β-D-thiogalactopyranoside (0.1 mM) was used to induce the expression of a glutathione transferase (GST)-MGARP fusion protein in Escherichia coli, and the protein was purified with an affinity column. A rabbit was inoculated with purified GST-MGARP fusion protein mixed with Freund’s adjuvant and boostered weekly for 4 wk. The serum of the rabbit was collected 1 wk later, and the purity and specificity of the antibody were analyzed by Western blotting and immunocytochemistry using anti-GST, preimmunized serum as control. The purified MGARP protein was used to do antigen preabsorption.

Identification of the female mice estrous cycle

A vaginal cast-off cell smear, hematoxylin and eosin (HE) staining method was used to identify the estrous cycle of female mice. A dipped wet cotton bud with 0.9% isotonic sodium chloride was inserted into the vagina and rotated several times. Samples were smeared onto slices and fixed with methanol. The vaginal cast-off cellular morphology was observed after HE staining.

GnRH antagonist experiment

ICR mice were divided into the following four groups: male control group, male GnRH antagonist group, female control group, and female GnRH antagonist group (n = 4–6/group). Mice in the GnRH antagonist groups were treated with daily sc injections of 0.5 mg/kg cetrorelix acetate (14) for 7 d, whereas the control groups were treated with the same volume of saline. The phase of the estrous cycle for all of the female mice was identified by the vaginal cast-off cell smear test before and after cetrorelix treatment.

Leptin intervention

ICR mice were divided into the following six groups: male control group, male leptin low-dose group, male leptin high-dose group, female control group, female leptin low-dose group, and female leptin high-dose group (n = 3/group). A dose of either 100 or 500 µg/kg leptin was given daily for 5 d by ip injection. The dose of 100 µg/kg leptin corresponds to the physiological dose (15), and 500 µg/kg of leptin corresponds to a hyperphysiological dose. The control group was treated with the same volume of saline. Euthanasia was performed on all mice (1% sodium pentobarbital). The phase of the estrous cycle for all the female mice was identified by the vaginal cast-off cell smear test before and after leptin treatment.

Immunohistochemistry

Tissues and organs, except for the eyeballs, from each adult mouse were fixed in 10% formalin for 24 h and embedded in paraffin. Eyeballs were fixed in a special fixative solution (glacial acetic acid:formalin:0.9% sodium chloride:75% alcohol, 1:2:7:10). Two serial 5-µm paraffin sections were used for immunohistochemical staining. Mouse MGARP antibody or GST antibody, used as a control, was added to the sections using a dropwise technique.

After the mice were euthanized, the tissues from each mouse were removed, fixed for 24 h, and embedded in paraffin. Then 5-µm sections were cut and stained using an immunohistochemical method to observe and analyze MGARP expression. Additionally, the same tissue types used for immunohistochemical staining were also taken from the contralateral side of each mouse for Western blotting.
Antigen preabsorption

Anti-MGARP antiserum was diluted (1:10,000) with purified MGARP-GST fusion protein dissolved in PBS with 1% BSA. The protein level of purified MGARP-GST fusion protein is 5.2 mg/ml (determined by bicinchoninic acid assay; Pierce, Rockford, IL). Anti-MGARP antiserum diluted (1:10,000) with PBS supplemented with 1% BSA was set as positive control. Both of which described above were incubated at 37°C for 1 h and followed by standard immunohistochemistry procedure. The tissue sections were also preincubated with 1% BSA in room temperature for 2 h.

Western blotting

Total protein was isolated from mice tissues. Tissues were collected and homogenized in protein extraction buffer [50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 1% Nonidet P-40, 1 mM EDTA, and 1% protease inhibitor cocktail]. The lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was collected. The supernatant (30 μg of protein) was resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. After being blocked with 5% nonfat milk, the membranes were probed with the primary mouse MGARP antibody (1:10,000) for 1 h, followed by a secondary antibody (goat antirabbit IgG horseradish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge). Protein expression was detected with an enhanced chemiluminescence detection system (Vigorous, Beijing, China).

Surgical procedures and estrogen treatment

Mice were weighed and anesthetized with 1% sodium pentobarbital by ip injection. Surgical castration was performed through the backside to gain bilateral access to the ovary. After the ovaries were removed, the skin was sutured with 3–0 vicryl. Mice in the sham group only had fatty tissue near the ovaries removed as a control. Ten days after the operations, mice were divided into the following four groups: control group with ovariectomy (OVX) (n = 5), OVX + E2 low-dose (E2-L) group (n = 6, 10 μg/kg), OVX + E2 high-dose (E2-H) group (n = 6, 100 μg/kg), and sham treatment group (n = 5). Estrogen (E2) was dissolved in 10% alcohol and 90% peanut oil (16) and given daily by sc injections for 1 wk. The dose of 10 μg/kg of E2 corresponds to the physiological dose, and 100 μg/kg of E2 corresponds to a hyperphysiological dose. Mice in the control group and sham group were sc injected with the same dose of vehicle (90% peanut oil and 10% alcohol).

Statistical analysis

Data are presented as the means ± se and were analyzed by ANOVA, followed by Tukey’s test for multiple comparisons or Student’s t test. Differences are considered significant when P < 0.05.

Results

MGARP is highly enriched in the steroidogenic and visual systems

The systemic study on the MGARP expression in adult mouse by immunohistochemistry demonstrated that, in addition to the retina, MGARP is highly expressed in all other parts of the eyeball, including cornea, lens, ciliary body, sclera, and choroid. There was robust expression in the corneal epithelial cells, scleral fibroblast cells, epithelial cells in the lens, and pigment epithelial layer of the ciliary body (Fig. 1A). The systemic expression profile also showed that MGARP is highly expressed in the ovary, testis, adrenal gland, eye, and brain, but not detectable in heart, liver, spleen, lung, kidney, skeletal muscle, fat tissue, stomach, small intestine, uterus, pancreas, prostate, thymus, parathyroid gland, pituitary gland, and thyroid gland by immunohistochemistry (Supplemental Fig. 1A, Group, Inc., Chicago, IL), followed by a secondary antibody (goat antimouse IgG horse radish peroxidase-conjugated antibody, 1:5000; Zhongshan Golden Bridge). Protein expression was detected with an enhanced chemiluminescence detection system (Vigorous, Beijing, China).
published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Using the antigen-adsorbed MGARP antibody, the staining was completely eliminated (Supplemental Fig. 1B). Further analysis indicated that MGARP was enriched in the lutein cells of corpus luteum, theca cells, and granulosa cells of ovarian follicles, Sertoli cells, and interstitial cells of mice testis as well as the zona glomerulosa, zona fasciculata, and zona reticularis of adrenal cortex. All these areas are made up by steroidogenic cells (Supplemental Fig. 2). Most importantly, our results showed that MGARP was detected in different regions of the brain, including the optic chiasma, optic tract (opt), lateral geniculate nucleus (LGN), laterodorsal thalamic nucleus ventrolateral part, and superficial gray layer of the superior colliculus (SuG) (Fig. 1B). All of these comprise the main components of the visual nervous system. Together, our observations indicate that MGARP is enriched in steroidogenic tissues and the visual system. Considering that the visual nervous system, including the retina and related areas in the brain, belongs to the central nervous system (CNS) as well as responds to hormones, we hypothesized that MGARP is potentially involved in the functional activities of the HPG axis.

The expression of MGARP during mouse development

To understand the role of MGARP in development that is critically regulated by the HPG axis, we studied MGARP expression during steroidogenic tissue development. As shown in Fig. 2, at postnatal d 1, MGARP is readily detected in the adrenal gland and only slightly detected in the retina. It was not detectable, however, in the mouse ovaries and testes at this stage. In the gonads, MGARP expression was clearly detected in pups 2–4 wk after birth (Fig. 2, A–C). In addition, we studied MGARP expression in more detail during the five phases of ovarian follicle development by immunohistochemistry. The expression of MGARP in the later phases was much higher than in the early phases (Supplemental Fig. 3).

The effects of estrogen on MGARP expression

To study whether the expression of MGARP is regulated by sex hormones and associated with steroidogenic activity of the HPG axis, we examined MGARP expression in female mice at different estrous cycle phases. We found that in ovaries, the expression of MGARP was significantly higher during estrus and diestrus than during proestrus and metestrus (Fig. 3, A–C). However, there was no significant fluctuation in MGARP expression in the mice adrenal glands and retinas at different estrous cycle phases (Fig. 3C).

To further determine the effects of sex hormones on MGARP expression, we generated OVX mice to reduce the endogenous estrogen or performed sc injection into the OVX mice with different doses of E2 to restore the estrogen level. The expression of MGARP was decreased in the retina of the OVX mice compared with the sham group, but the magnitude is not statistically significant, whereas injection of E2 at a dose of 100 μg/kg could increase the expression of MGARP by 25% compared with the OVX group (Fig. 3D). In contrast, the expression of MGARP in the adrenal gland was increased in the OVX mice compared with the sham group. Furthermore, injection of E2 at a dose of 100 μg/kg could reduce the expres-
sion levels of MGARP by 21% in the adrenal gland compared with the control of OVX group (Fig. 3E).

The effects of functional inhibition of GnRH on MGARP expression

Next, we studied MGARP expression by manipulation of GnRH, a key factor in the HPG axis. Cetrorelix was used in these tests, which is a GnRH antagonist that competes the binding of GnRH to its receptors to inhibit gonadotropin level. The staining of MGARP in the cytoplasm of Leydig cells and Sertoli cells of mice testes was reduced under the treatment of cetrorelix (GnRH antagonist) compared with the untreated control. Its expression in follicular cells of ovary was also reduced, but it is not as significant as that occurring in treated testes (Fig. 4A). As shown by Western blotting, cetrorelix treatment led to the reduction in MGARP expression by 62% in testes and 33% in ovaries compared with the control groups (Fig. 4, A–C). However, no difference was observed in the retina and adrenal gland.

The effects of leptin on MGARP expression

To further examine a role of the HPG axis in regulating MGARP expression, we investigated the effects of leptin, which has been reported to stimulate the GnRH secretion through acting on the hypothalamus (17). As shown in Fig. 5, A and B, exogenous injection of leptin into female mice did not induce an obvious change in MGARP expression in the ovaries. In the follicular granular cells of the sinusoid follicle, however, a low-dose of leptin (100 μg/kg) clearly increased MGARP expression compared with the control. To discount the effect of the estrous cycle on MGARP expression, we carried out similar studies by using male mice. Injection of exogenous leptin at a dose of 100 μg/kg into adult male mice also increased MGARP expression by 45% in the testes compared with the control, with a particularly higher stimulation in Leydig cells (Fig. 5, C and D).

Another model used in this study is the female leptin knockout (ob/ob) mice, which have been demonstrated to be infertile (18). We studied the expression of MGARP in ovaries of ob/ob mice and wild-type C57 mice by immunohistochemistry. Consistently, the overall expression of MGARP in ovaries of the ob/ob mice did not show significant differences, but its expression in follicular granular cells was markedly reduced compared with the wild-type control mice (Fig. 5, E and F).

Discussion

We previously identified the MGARP gene by a microarray from the mouse retina (11). Retina, a particularly accessible part of the CNS, is critical for the capture of images and the response to light. These processes require the...
collaboration of several different parts of the eyeball. To understand a potential role of MGARP in the visual system, we tested for MGARP expression in each part of the eyeball by immunohistochemistry. The results demonstrated that, in addition to the retina, MGARP is also highly expressed in all other parts of the eyeball. To gather more information about MGARP, we conducted a systemic analysis of MGARP expression by immunohistochemical staining. The results showed that MGARP is highly expressed in steroidogenic and nervous tissues, especially in areas that make up the main components of the visual nervous system. The optic chiasma, opt, and LGN are the main components of the visual pathway in brain. These regions are responsible for transmitting visual information from the eyeball to the optic center in the brain. The superficial gray layer of the superior colliculus is responsible for providing a powerful excitatory input to the intermediate layer, which plays a critical role in initiating rapid orienting movements of the eye (19–23). Thus, most of the regions positive for MGARP expression are directly or indirectly involved in visual processing. The visual system is a special system that has long been used as a model for CNS developmental and functional studies of sex hormone (estrogen, progesterone, and androgen) actions. Various physiological conditions, such as age, menstrual cycles, pregnancy, and menopause or andropause can affect vision (24). Studies have also shown the presence of sex steroid hormone receptors in various ocular tissues, such as the lens, retina, choroid, cornea, and ciliary body, and the response of the retina to sex steroid hormone action is similar to that of the CNS (24). With in situ hybridization techniques, estrogen receptor mRNA was detected in both the retina and brain (25, 26). The retina was also able to synthesize steroid hormones by the progesterone pathway (27). Considering the CNS (brain and spinal cord) and retina are steroidogenic tissues (27–29), and steroid hormones are regulated by the HPG axis, our findings suggest a profound role of MGARP in the regulatory loop of the HPG axis.

However, why is MGARP predominately expressed within the visual system? This is a critical question that is worth of further study. Here, we propose several reasons. 1) The visual system has higher demand for both energy and steroid hormones. 2) As part of the CNS, the visual system is the major regions of the animal body to sense or receive the stimuli from the environment. The visual perception is not simply a translation of stimuli and formation of the image on the retina and it should need the eye and the related visual tissues in the brain to work together in a very complicated way. This is a question scientists in this field have long struggled to explore. Our observation that MGARP is highly expressed in visual system implies that MGARP may play critical function in transmitting the stimuli through responding to the steroid hormones or through regulating hormone synthesis. 3) The expression difference in the brain reflects that MGARP expression is under the regulation of different hormones in a direct or indirect manner, because different regions of the brain produce distinct hormones and those hormones always function in different tissues through circulation. It can be considered that HPG axis may steer the entire process through regulating the expression level of MGARP. Certainly, all these need more studies to be clearly addressed.

The HPG axis plays an active and essential role in mammalian development via modulating various hormones.
Observations of MGARP expression in mice retinas and adrenal glands during mouse development indicate that MGARP plays an early role in the development of the organs. In the gonads, MGARP expression was clearly detected in pups only 2–4 wk after birth. We speculate that as the levels of sex hormones increase in the gonads, they stimulate the expression of MGARP. The increased expression of MGARP, in turn, directs the biosynthesis of distinct sex hormones in the gonads and facilitates the development of the sex organs.

During the development of mice ovaries, many ovarian follicles are involved in the process of maturation, whereas only a few of them named dominant follicles will eventually develop into mature ova (30). It has been reported that MGARP mRNA is up-regulated in dominant follicles compared with the subordinate follicles, and MGARP is less expressed in the early phase of follicular development than in other phases (31). Our results of the MGARP expression during the five phases of ovarian follicle development indicate that MGARP expression is gradually increased during follicle development, which is most likely regulated by gonadotropin.

One physiological function of the HPG axis is to regulate the synthesis of sex hormones. Conversely, the sex hormones regulate the HPG axis through a feedback regulatory mechanism. Estrogen is a sex hormone that not only affects the sex organs, but it also affects the structure and function of the nervous system, because its receptors are expressed in the brain regions that are involved in sex differentiation and maturation (1). To determine whether the expression of MGARP is involved in the steroidogenic activity of the HPG axis, we used a female mouse model, because they have a clear estrous cycle that is linked to the endogenous fluctuations of sex hormones. In estrous cycles, estrogen and progesterin levels are dominant during estrous and diestrous phase, thus, the MGARP expression change in ovaries at estrous cycle suggests that MGARP expression is correlated with the levels of estrogen and progesterin in the ovary. It is reasonable that no significant change in MGARP expression can be found in the adrenal gland and retina during the estrous cycle, because the estrous cycle mainly affects the fluctuation of sex hormones in the gonads. Together, our findings indicate that the expression of MGARP is regulated by steroid hormones in a tissue-specific manner.

OVX mice provide a model to monitor the effects of estrogen under a reduced background. Our results indicate that estrogen in OVX mice exerts opposite effects on MGARP expression in the retina and the adrenal gland, further suggesting a tissue-specific regulatory mechanism that controls MGARP expression. The decreased expression of MGARP in the retina is likely due to a direct effect of estrogen reduction after removal of the ovaries. Conversely, the increased expression of MGARP in the adrenal gland may be due to the negative feedback mechanism in the HPG axis that regulates estrogen levels (32). Simi-
Leptin can stimulate GnRH secretion through acting on the hypothalamus (35). Our results showed that exogenous injection of leptin into female mice clearly increased the MGARP expression in follicular granular cells of antral follicle, especially under the treatment of lower dose of leptin (100 µg/kg). Similarly, exogenous injection of leptin into male mice could also stimulate MGARP expression by 45% in the testis, much higher than in ovary. These results suggest that leptin can induce the MGARP expression by stimulating the secretion of GnRH via the HPG axis.

In summary, our study revealed a regulatory loop mechanism that exists between MGARP expression and steroidogenesis in the HPG axis. Additionally, we demonstrated that MGARP is highly expressed both in the visual nervous system and steroidogenesic tissues. MGARP is regulated in a development-dependent and tissue-specific manner. This also implies that an animal’s body keeps the balance of hormone levels by combinatory mechanisms that are both feedback regulatory and tissue-specific. The coordination between steroid hormones and neuronal control regulates important events within the animal body, including organogenesis, growth, aging, the

**FIG. 6.** Model demonstrating the potential role of MGARP in the HPG axis. Solid arrows represent known pathways, whereas dashed arrows represent proposed pathways. STAR, Steroidogenic acute regulatory protein; PKA, protein kinase A.
stress response, and the clearing of most diseases. Thus, this finding demonstrating the involvement of MGARP in the HPG axis will be of great biological and clinical significance.

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Address all correspondence and requests for reprints to: Shuping Zhang, School of Life Sciences, Tsinghua University, Beijing 100084, China. E-mail: bczhang@tsinghua.edu.cn.

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