Original Article

Immobilization stress increased cytochrome P450 1A2 (CYP1A2) expression in the ovary of rat

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ABSTRACT Under the stressed condition, a complex feedback mechanism for stress is activated to maintain homeostasis of the body and secretes several stress hormones. But these stress hormones impair synthesis and secretion of the reproductive hormones, followed by suppression of ovarian function. Cytochrome P450 1A2 (CYP1A2) plays a major role in metabolizing exogenous substances and endogenous hormones, and its expression is recently identified at not only the liver but also several organs with respect to the pancreas, lung and ovary. Although the expression of CYP1A2 can be also affected by several factors, understanding for the changed pattern of the ovarian CYP1A2 expression upon stress induction is still limited. Therefore, CYP1A2 expression in the ovaries from immobilization stress-induced rats were assessed in the present study. The stress-induced rats in the present study exhibited the physiological changes in terms of increased stress hormone level and decreased body weight gains. Under immunohistological observation, the ovarian CYP1A2 expression in both control and the stressed ovary was localized in the antral to pre-ovulatory follicles. However, its expression level was significantly ($p < 0.01$) higher in the stress-induced group than control group. In addition, stress-induced group presented more abundant CYP1A2-positive follicles (%) than control group. Since expression of the ovarian CYP1A2 was highly related with follicle atresia, increased expression of CYP1A2 in the stressed ovary might be associated with changes of the ovarian follicular dynamics due to stress induction. We hope that these findings have important implications in the fields of the reproductive biology.

Keywords: cytochrome P450 1A2, immunohistochemistry, ovary, stress

INTRODUCTION

Under the stressed condition, a complex feedback mechanism of the hypothalamo-pituitary-adrenal (HPA) axis is activated to regulate interactions to stress to maintain homeostasis of the body (Iwasa et al., 2018; Kim,

2019). In particular, the hormones in HPA axis with respect to corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoid reduce the pulsatile secretions of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in hypothalamo-pituitary-

adrenal (HPG) axis of animals, followed by impairments of ovarian cyclicity and synthesis/secretion of gonadotropin (FSH and LH) (Gai et al., 2016; Kim, 2019). Regarding the impairments by stress, several stressors in the rodent attenuate the ovarian cyclicity in regards to less number of secondary and antral follicles, and atresia induction in developing follicles (Wu et al., 2012; Kim, 2019). In case of alteration of reproduction hormone due to stress, because estradiol plays a key role in regulating the follicle development, ovarian atresia and growth of granulosa cells in the developing follicles, its decrease caused by stress is critical in the normal ovarian cyclicity and fate of granulosa cells (Li et al., 2016; Kim, 2019).

Cytochrome P450 (CYP) superfamily is a physiologically key enzyme for the biotransformation and metabolism of endogenous substances and exogenous chemicals (Dey et al., 1999; Lu et al., 2020). And it can be classified as CYP family, its subfamily and their isoform (McDonnell and Dang, 2013). In particular, cytochrome P450 1A2 (CYP1A2) plays a major role in biotransformation of a wide range of exogenous substances such as chemicals, pollutants and drugs as well as endogenous hormones with respect to progesterone, estrone and estradiol in humans (Dey et al., 1999; Mikhailova et al., 2006; Sugiyama et al., 2019; Lu et al., 2020). It has been known that CYP1A2 is mainly expressed in the liver to metabolize the aromatic amines and heterocyclic compounds of exogenous substances and endogenous hormones (Mikhailova et al., 2006; Sugiyama et al., 2019; Lu et al., 2020). However, in the recent days, it has been verified that CYP1A2 is also expressed in various organs in terms of the pancreas, lung and ovary (Hong et al., 2004; Vukovic et al., 2016; Hwang et al., 2020). Especially, it is found that expression of CYP isoforms in the ovary can be altered under 3-methylcholanthrene (3MC; Ah receptor ligand) treatment, the onset of puberty, methoxychlor (MXC; estrogenic endocrine disruptor) application (Dey et al., 1999; Symonds et al., 2006; Hwang et al., 2020).

Since the female exposed to several stressors is easy to be attenuated to their reproductive function in the hormone level (down regulations of gonadotropins and estrogen) and ovarian cyclicity, stressors may also change the expression pattern of CYP1A2 in the ovary (Yang et al., 2017; Kim, 2019). However, the relationship between stress induction and CYP1A2 expression in the ovary is not understood well, in spite of the fact that the ovary

functions can be affected by several factors (Hwang et al., 2020). In addition, most researches for investigating function of CYPs and alterations of their expression pattern under a certain stimulus are mainly concentrated on the liver (McDonnell and Dang, 2013). Therefore, the present study was aimed to reveal the expression of CYP1A2 in the ovary of rats in response to immobilization stress, in order to clarify the effect of stress to the ovarian CYP1A2 (Hwang et al., 2020).

MATERIALS AND METHODS

Ethics statement

All procedures for animal experiments were approved by the Institutional Animal Care Use Committee at Kyungpook National University (approval number: KNU2019-0159).

Chemicals and media

All chemicals and media were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise specified.

Experimental groups

The 32-day-old female Sprague-Dawley (SD) rats (mean body weight: 57.6 g; total n = 16) were used in the present study. The arrival day of animals was regarded as Day 0. During the experiment, the animals were housed in the plastic cages (40 × 32 × 17 cm; 3–4 animals/cage), maintained for temperature at 23 ± 2°C, humidity at 50–80% and approximately 12 h light/dark cycle. The standard feed (Jeil Feed Co., Ltd., Korea) and tap water were provided *ad libitum*. Immobilization stress from Day 1 to Day 20 was daily induced for 6 h from 06:00 to 12:00 using the rat strainer as the stress group (STR, n = 8), in accordance with the previous study; the control group (CON, n = 8) was treated in a same manner with STR group, except for immobilization stress induction (Darnaudéry and Maccari, 2008; Kim, 2019).

Sacrifice of animals and sampling

Both groups were sacrificed at Day 20 upon finishing the last immobilization stress induction of STR group. Body weight (BW) was measured using a weighing machine at Day 1 and just before sacrifice (Day 20). The animals were anesthetized with isoflurane inhalation in the closed chamber and exsanguinated via the right atrium.

During exsanguination, the whole blood for stress hormone assays was collected in ethylenediaminetetraacetic acid (EDTA) tube (BD falcon, NY, USA). Serum was gently isolated from supernatant of whole blood by centrifugation at 4,000 rpm for 15 min in 4°C, followed by storage in a deep freezer at -80°C until using. The ovary from both groups were collected, trimmed, snap-frozen into liquid nitrogen (LN₂) and stored in a deep freezer (-80°C) for western blotting (each n = 4) or fixed with 4% paraformaldehyde (Duksan chemical, Korea) for immunohistochemistry (IHC) (each n = 4). In addition, after isolating the brains from the skulls, the hypothalamus that located undersurface of the brain, and lay just below the thalamus and above the pituitary gland was bluntly dissected using micro forceps. Then the hypothalamus was treated in a same manner with ovaries for western blotting.

Hormone assay by enzyme-linked immunoabsorbent assay

Serum level of corticosterone was measured by enzyme-linked immunoabsorbent assay methods (ELISA; Cayman Chemical Company, MI, USA). Stored serum in a deep freezer was immediately used upon thawing. Enzyme-immunoassay (EIA) buffer, wash buffer, tracer and antiserum were prepared in advance, following the manufacturer's instruction. Mixture of sample, EIA buffer, tracer and antiserum was incubated for 90 min at room temperature (RT). Thereafter, samples on the 96-well plate were reacted with reagents for 1 h in the incubator. The reacted 96-well plate was read at a wavelength in 405 nm using a microplate reader (Epoch, Biotek, VT, USA). The concentration of corticosterone in the serum of both groups was calculated by a 4-parameter logistic fit using free software (www.myassay.com).

Western blotting

The western blotting for CYP1A2 expression on the ovary or CRH expression in the hypothalamus was conducted in accordance with the previous articles (Kim, 2019; Hwang et al., 2020). In brief, the snap-frozen ovaries or hypothalami was homogenized with a homogenizer, lysed with a radioimmunoprecipitation assay (RIPA) buffer supplemented a proteinase inhibitor and centrifugated at 14,000 rpm for 5 min at 4°C. Thereafter, supernatants were carefully isolated and quantified for the total amount of protein by means of a protocol with a Bicinchoninic

Acid Protein Assay Reagent Kit. The total proteins (10 µg) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Then the membranes were blocked with 3% bovine serum albumin (BSA) for 1 h at RT and incubated with a mouse polyclonal anti-CYP1A2 antibody (1:500 dilution with 1% BSA; Santa Cruz Biotechnology, TX, USA) in the ovary or a rabbit polyclonal anti-CRH (1:500 dilution with 1% BSA) in the hypothalamus for overnight at 4°C. As reference, a mouse polyclonal anti-Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; 1:1,000 dilution with 1% BSA) was employed in both samples. The membranes were incubated with a horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG (1:3,000 dilution with tris buffer saline containing 0.1% tween-20) for 1 h at RT and developed on X-ray films (AGFA, Belgium) using an enhanced chemiluminescence (ECL) kit. Image J software (National Institutes of Health, USA) was involved for quantification of the intensities of developed bands. The expression level of CYP1A2 or CRH was relatively normalized against that of GAPDH.

Immunohistochemistry in the ovary

The procedure for IHC was followed with the previous articles (Kim, 2019; Hwang et al., 2020). In brief, the fixed ovaries from each group were dehydrated, embedded in paraffin and sectioned into 5 mm thick using a microtome (Leica Microsystems, Germany). Then the slides were deparaffinized, treated with 0.01 M citrate buffer (pH 6.0) at 95°C for 30 min, cooled at RT for 60 min, treated with 3% H₂O₂ for 30 min, washed with phosphate buffered saline with 0.1% triton-X (PBS-T), blocked with 2% normal horse serum (Vector Laboratories, CA, USA) for 1 h at RT and incubated with a mouse polyclonal anti-CYP1A2 antibody (1:50 dilution with 2% normal horse serum) at 4°C for overnight. Thereafter, the Histostain-plus kit (Vector Laboratories) was applied to incubate slides with a biotinylated secondary antibody (1:200 dilution with PBS) at RT for 90 min and treat slides with an ABC solution at RT for 60 min. All slides were reacted with a 3,3'-diaminobenzidine (DAB) kit (Vector Laboratories) for the same amount of time and counterstained with hematoxylin. The brownish color on the tissue was determined as the positive expression of CYP1A2 under the microscopic observation (Moticam Pro 20A, Motic, Hong Kong). During

observation of the CYP11A2-positive follicles, the follicles were classified in regards to the primary follicles (a single layer of cuboidal granulosa cells), secondary follicles (surrounding of more than one layer of cuboidal granulosa cells without visible antrum), antral follicles (multiple layer of granulosa cells and 1-2 small spaces of antrum) and pre-ovulatory follicles (the largest follicle with cumulus granulosa cell layer surrounding the oocyte).

Statistical analysis

Mann-Whitney U test was applied to determine a significant difference between groups using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). A p value of < 0.01 was considered to be statistically significant.

RESULTS

Characterization of stress-induced animals

Immobilization stress was induced in STR group to in-

vestigate the alteration of CYP11A2 expression in the ovary in the present study. To characterize stress induction-related physiological changes, western blotting for CRH expression in the brain, ELISA for quantification of corticosterone in the serum and measurement of BW gains during experimental period (Day 1 to Day 20) were performed (Fig. 1). The expression of CRH, upstream stress-related hormone, was significantly ($p < 0.01$) elevated in the brain of STR group (Fig. 1A and 1B). Consistent with CRH expression, STR group presented significantly ($p < 0.01$) higher level of corticosterone, downstream stress-related hormone, in the serum (Fig. 1C). In case of BW gains, there was a significant ($p < 0.01$) change between CON and STR groups (Fig. 1D). Collectively, it was determined that the protocol of immobilization stress in the present study could successfully induce the stress-related changes in the body.

Fig. 1. Characterization of stress-induced animals. The CRH expression in the hypothalamus, upstream stress hormone, was assessed by western blotting (A and B). At the serum level, the concentration of corticosterone, downstream stress hormone, was evaluated with enzyme-linked immunoabsorbent assay (ELISA) assay (C). Body weight gains during experimental period were compared between Con and STR groups (D). *Superscript indicates a significant difference between groups ($p < 0.01$). CON: control group, STR: immobilization stress-induced group, CRH: corticotrophin releasing hormone, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Western blotting for CYP1A2 expression in the ovary

The western blotting was conducted to assess quantitative expression of CYP1A2 in the ovaries of CON and STR. The expression of CYP1A2 in the ovary of STR group was significantly ($p < 0.01$) increased under immobilization stress (Fig. 2). Given that stress induction took place to follicle atresia (Kim, 2019) and CYP1A2 expressions were

Fig. 2. Western blotting for CYP1A2 expression in the ovary. The expression of CYP1A2 in the ovary was assessed by western blotting (A) and quantitatively compared between groups (B). Graph was presented as mean $\pm$ SEM. *Superscript indicates a significant difference between groups ($p < 0.01$). CYP1A2: cytochrome P450 1A2.

localized in the atretic follicles (Hwang et al., 2020), the present finding suggested that immobilization stress induced the follicle atresia, followed by increase of CYP1A2 expression in the ovary.

Immunohistological expression of CYP1A2 in the ovary

The expressions of CYP1A2 in the ovaries of both groups were assessed by IHC (Fig. 3). Although both groups expressed CYP1A2 at the cells surrounding follicles (granulosa cell layers), the patterns were dependent on the stage of developing follicle and the stress induction. Consistent with the previous results that CYP1A2 mainly expressed in the antral and pre-ovulatory follicles (Hwang et al., 2020), the expression of CYP1A2 of both CON and STR groups was undetectable in the primordial, primary and secondary follicles (red arrows in Fig. 3A and 3D); from the antral to pre-ovulatory follicles, the expression of CYP1A2 was observed in the granulosa cell layers (blue arrows in Fig. 3B, 3C, 3E and 3F). When the CYP1A2-positive follicles were counted (%), CYP1A2-positive follicles were significantly ($p < 0.01$) more abundant in STR than CON group (Table 1).

Table 1. Counting (%) for CYP1A2-positive follicles in the ovaries

	CON (n = 4)	STR (n = 4)
CYP1A2-positive follicle (%)	73.0 $\pm$ 4.3	83.6 $\pm$ 3.5*

The values are described as mean $\pm$ SD. *Superscript indicates significant difference between groups ($p < 0.01$).

Fig. 3. Immunohistological expression of CYP1A2 in the ovary. The expression of CYP1A2 in the ovary was assessed by immunohistochemistry (IHC). At both groups, the primordial, primary and secondary follicles exhibited the negative expression of CYP1A2 (red arrows in Fig. 3A and 3D). The CYP1A2-positive cells were observed from antral follicles and pre-ovulatory follicles (blue arrows in Fig. 3B, 3C, 3E and 3F) and they were localized at the granulosa cell layer. Magnification: $\times 400$ (3A and 3D) or $\times 100$ (3B, 3C, 3E and 3F); Bars: 100 μ m (3A and 3B) or 1,000 μ m (3B, 3C, 3E and 3F).

Collectively, these results indicated that the expression of CYP1A2 in the ovary was elevated by immobilization stress.

DISCUSSION

The studies of CYP1A2 expression are mainly focused on the liver to understand its role with respect to biotransformation and metabolism of exogenous substances and endogenous hormones (Mikhailova et al., 2006; Sugiyama et al., 2019). In the recent days, CYP1A2 expression in the other tissues including the pancreas, lung and ovary has been investigated (Vukovic et al., 2016; Hwang et al., 2020). Especially, there have been several articles identified that the expression of CYP family in the ovary is affected by several internal or external factors. The expression of ovarian CYP1B1 was increased under estrogen treatment (Symonds et al., 2006) or during evening of proestrus than other estrus cycles for metabolism of serum estradiol (Dasmahapatra et al., 2002). In addition, the expressions of CYP2E1, CYP2A and CYP2B were dependent on the follicle size of the ovary (Cannady et al., 2003). Furthermore, the ovarian CYP1A1 was increasingly expressed after treatment of 3MC and dexamethasone in the pig (Leighton et al., 1995) or exposure of 3MC in the mouse (Dey et al., 1999). In case of CYP1A2 expression in the ovary, the intensity of expression was dependent on the onset of puberty and its expression was localized in the atretic follicles of antral and pre-ovulatory follicle stages (Hwang et al., 2020). However, it is determined that the understanding for the changed pattern of the ovarian CYP1A2 expression upon internal or external factors is still limited. Thus, because external factors like stress could alter the physiology of female reproductive system, the effect of immobilization stress into the expression of the ovarian CYP1A2 was investigated in the present study. Here, we demonstrated that CYP1A2 expression in the stressed ovary was localized in the antral to pre-ovulatory follicles, consistent with control group (Fig. 3); however, its expression in the follicles of stress group was more abundant than that of control group (Fig. 2 and Table 1). In accordance with the previous articles, CYP1A2 expressions in the ovary were specifically localized Fas-positive follicles, indicating the atretic follicle; the increase of CYP1A2 expression in the atretic follicle was regarded as the reactions for metabolizing residue of hormones in the follicle (Hwang et al., 2020). In addition, the chronic and

unpredictable stress induced the follicle atresia, followed by suppression of ovarian cyclicity in the mouse (Kim, 2019). Therefore, results from the present study, increased CYP1A2 expression in the stressed ovary, might be explained as a response for immobilization stress-induced atresia in the follicles.

Stressors impair several reproductive capacities in female with respect to weakening ovarian cyclicity, change of synthesis/secretion upstream gonadotropin, alteration of reproduction hormones and fate of developing follicles (Gai et al., 2016; Li et al., 2016; Kim, 2019). The present study demonstrated stress-related the physiological changes in the body in terms of increase of stress-related hormones, decrease of body weight gains and imbalance of reproductive hormones (Fig. 1). Likewise, there have been clear evidences that stress can affect to physiological changes, followed by attenuation of reproductive capacity and alteration of CYPs expression in the female. Heat stress induced alteration of the ovarian follicular dynamics and granulosa cell function, and diminished reproductive capacity via reduction of estrogen concentration, the number of atretic follicles and CYP19 (aromatase) expression in mouse (Li et al., 2016). In addition, chronic and unpredictable stress in the mouse suppressed kisspeptin expression in the hypothalamus, an upstream regulator of HPG axis, resulted in irregularly shrunk oocytes with broken zona pellucida throughout the follicle stages, pyknotic granulosa cells, decreased number of developing follicles and increased number of atretic follicles in the ovary (Kim, 2019). And other stressors such as serum deprivation, tunicamycin (endoplasmic reticulum stressor), nanosilver particles, reactive oxygen species (ROS) and organic pollutant (polycyclic aromatic hydrocarbons) could induce granulosa cell apoptosis, impaired angiogenesis into the follicle, diminished estrogen concentration, decrease of CYP19 expression and increase of pro-apoptotic proteins in the ovary (Lin et al., 2012; Mirzaei et al., 2017; Yang et al., 2017; Sheng et al., 2018). Likewise, stressors are highly associated with the ovary functions with apoptosis of granulosa cells in the atretic follicles. However, except for CYP19, it has been still not understood well whether stress can affect on expressions of other types of the ovarian CYP family. Here, we demonstrated that immobilization stress in the rats increased the ovarian CYP1A2 in the antral and pre-ovulatory follicles, compared to control (Fig. 2, 3, and Table 1). Since CYP1A2 is highly related

with follicle atresia (Hwang et al., 2020), its alteration in the ovary may be associated with changes of the ovarian follicular dynamics due to stress induction.

Likewise, investigations of expression and role of CYP1A in the body are meaningful in the field of biology for understanding not only reproductive biology but also hormone-related disorders; because CYP1A has been known for capacity of metabolizing certain substances including gender hormones, its alteration may take place to hormone imbalance, followed by occurring certain diseases such as reproductive disorders and cancers (Hong et al., 2004; Kim, 2019; Hwang et al., 2020). This alteration includes the genetic diversity of CYP1A, called as the single nucleotide polymorphisms (SNPs) derived by single nucleotide variation; in fact, the number of SNPs in CYP1A1 or CYP1A2 is addressed as 27 or 48 in human, respectively (Lu et al., 2020). In particular, imbalance of gender hormone (estrogen) due to SNPs of CYP1A and CYP1B in human is related with several disorders with respect to hypertension, age-related macular degeneration, breast cancer, ovarian cancer, testis cancer and risk of infant birth size (McKay et al., 1995; Shin et al., 2007; Diergaarde et al., 2008; Sergentanis and Economopoulos, 2010). Therefore, although it has been already known that the hepatic CYP1A2 mainly works in circulating hormones in the serum, it is an imperative to continue to further study of CYP1A2 roles in other organs in order to fully understand the etiology of CYP1A2-related hormonal disorders or discover a predictor of certain disease (Hong et al., 2004).

In conclusion, the present study hypothesized that CYP1A2 expression in the ovary could be affected by stress induction because ovarian cyclicity was tightly altered under external factors. Taken together, we demonstrated that CYP1A2 expression was elevated in the stressed ovary, which might be considered as a response for changes of the reproductive hormone. We hope that these findings have important implications in the fields of the reproductive biology.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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