Heat stress diminishes gonadotropin receptor expression and enhances susceptibility to apoptosis of rat granulosa cells

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Abstract

Heat stress inhibits ovarian follicular development in mammalian species. We hypothesized that heat stress inhibits the function of follicular granulosa cells and suppresses follicular development. To test this, immature female rats were injected with pregnant mare serum gonadotropin (PMSG) at 48 h after the start of temperature treatment (control: 25 °C, 50% RH; heat stress: 35 °C, 70% Relative Humidity). The ovaries and granulosa cells of follicles at different developmental stages were analyzed for gonadotropin receptor levels and aromatase activity; estradiol levels were measured in follicular fluid. Before injection, heat stress diminished only the amount of FSH receptor on granulosa cells of antral follicles. During PMSG-stimulated follicular development, heat stress strongly inhibited gonadotropin receptor levels and aromatase activity in granulosa cells, and estradiol levels in the follicular fluid of early antral, antral and preovulatory follicles. To examine apoptosis and mRNA levels of *bcl-2* and *bax* in granulosa cells, follicles harvested 48 h after PMSG injection were cultured in serum-free conditions. Heat-stressed granulosa cells showed a time-dependent increase in apoptosis. The *bcl-2* mRNA levels were similar in control and heat-stressed granulosa cells; *bax* mRNA levels were increased in heat-stressed granulosa cells. According to these results, heat stress inhibits expression of gonadotropin receptors in granulosa cells and attenuates estrogenic activity of growing follicles, granulosa cells of heat-stressed follicles are susceptible to apoptosis, and the *bcl2/bax* system is not associated with heat-stress-induced apoptosis of granulosa cells. Our study suggests that decreased numbers and function of granulosa cells may cause ovarian dysfunction in domestic animals in summer.

Reproduction (2005) **129** 463–472

Introduction

Heat stress inhibits ovarian follicular development in mammals such as domestic animals and rodents, and this can contribute to diminished reproductive efficiency during summer. In domestic animals, heat stress has been found to reduce the size of the dominant follicle by day 8 (Badinga *et al.* 1993) and between days 11 and 21 of the estrus cycle (Wilson *et al.* 1998a,b). In rodents, heat stress promoted follicular atresia and resulted in a decreased number of oocytes ovulated in immature female rats primed with pregnant mare serum gonadotropin (PMSG) (Shimizu *et al.* 2000).

During follicular development, the dominant follicles acquire high estrogenic activity compared with other follicles. Follicular estrogenic activity depends on the capacity for estrogen production of the granulosa cells (Fortune 1994). Estrogen production is stimulated by the binding of follicle-stimulating hormone (FSH) to its

receptors on the granulosa cell membrane, activating the aromatase enzyme that converts testosterone to estradiol (Dorrington *et al.* 1975, Erickson and Hsueh 1978). In turn, the synthesized estradiol contributes to the maintenance and expression of receptors for luteinizing hormone (LH) and FSH (LH-R and FSH-R respectively) on granulosa cells (Hsueh *et al.* 1994, Bodensteiner *et al.* 1995). Heat stress was found to suppress aromatase activity in granulosa cells and decrease the estradiol concentrations in the follicular fluid and plasma of dairy cows (Badinga *et al.* 1993). In addition, in both lactating cows and heifers, heat stress decreased serum estradiol concentration between days 11 and 21 of the estrus cycle (Wilson *et al.* 1998a,b). Seasonal and acute heat stresses were found to affect steroid production in the dominant follicles of cows (Wolfenson *et al.* 1997). These findings demonstrated that heat stress affects the viability of granulosa cells and the follicular estrogenic activity. However, the mechanism by

which heat stress suppresses estrogen production has not been investigated.

Follicular cells in atretic follicles are normally eliminated by apoptosis (Tilly 1993, Palumbo and Yeh 1994), as can be observed in granulosa cells during follicular development (Tilly 1996). Apoptosis of granulosa cells is provoked by the lack of survival factors such as FSH and estradiol (Billing *et al.* 1993, Chun *et al.* 1996). Although it has been shown that heat stress suppresses estradiol production in the dominant follicle (Badinga *et al.* 1993), it is unknown whether this suppression results in granulosa cell apoptosis. Recently, the expression of genes encoding apoptosis-regulating proteins in the follicle has been reported. Two members of the *bcl-2* family, *bcl-2* and *bax*, have been shown to regulate apoptosis in the ovary (Tilly *et al.* 1995, Adams and Cory 1998, Goodman *et al.* 1998). In the rat ovary, Bax appears to antagonize the apoptosis-suppressive effects of Bcl-2, and the fate of the cell appears to be decided by the balance between these two regulatory proteins (Tilly *et al.* 1995). In addition, previous study has reported that the ability of gonadotropins to prevent apoptosis and atresia in ovarian follicles may be linked to a shift in the ratio of *bcl-2* to *bax* gene expression (Tilly *et al.* 1995).

The objective of the present study was to determine whether heat stress affects the estrogen production system and whether granulosa cells in follicles isolated from heat-stressed immature female rats have an increased susceptibility to apoptosis. In addition, we investigated possible cellular mechanisms for the apoptosis induction of granulosa cells from heat-stressed animals.

Materials and Methods

Animal and heat stress treatment

Three-week-old immature Wistar female rats (Charles River Inc., Tokyo Japan) were used. The animals were divided into two groups (control and heat stress), kept in separate cages, and housed in a climate-controlled chamber at 25°C and 50% relative humidity, with a 12:12 h light:dark cycle (lights on at 0600 h). Each animal received a subcutaneous injection of 20 IU of PMSG (Teikoku Hormone Co., Tokyo Japan) at 48 h after the start of the temperature treatment. The heat stress group was exposed to 35°C and 70% relative humidity conditions from 48 h before PMSG injection to 48 h after injection (for 4 days). The animals were allowed rat chow (MF pellet, Charles River Inc.) and water *ad libitum*.

Follicle isolation and extraction of granulosa cells

The ovaries were removed from the rats either before or 48 h after injection of PMSG. The isolation and size classification of the follicles from the ovaries were performed by the methods of Roy and Greenwald (1985). The ovaries were separated by fine forceps and incubated in Medium

199 with 25 mM Hepes containing type I collagenase, DNase (bovine pancreas type III) and protease, in addition to 1% bovine serum albumin (BSA) (all purchased from Sigma) for 40 min at 37°C. Subsequently, Medium 199 containing 1% BSA was added, and the mixture was centrifuged at 600 r.p.m. for 4 min. The supernatant was discarded, and the pellet was resuspended in fresh Medium 199. The dispersed follicles were then separated by successive passage through nylon mesh of appropriate pore sizes to yield follicles with diameters of <185 µm (preantral follicles), 186–350 µm (early antral follicles), 351–500 µm (antral follicles) and >500 µm (preovulatory follicles). The granulosa cells and follicular fluid were separated from the collected follicles of different developmental stages. The number of each type of granulosa cells was counted on a hemocytometer and adjusted to 2.5×10^5 cells/100 µl.

Receptor binding assays

Highly purified rat FSH (NIDDK-rFSH-I-8) and LH (NIDDK-rLH-I-9) were radioiodinated by the lactoperoxidase method. Single-point saturation analysis was used to determine the numbers of FSH and LH receptors in granulosa cells, as described previously (Kawate *et al.* 1990).

¹²⁵I-rFSH binding assay

Aliquots (100 µl) of granulosa cells were incubated for 3 h at 37°C with a single dose of ¹²⁵I-labeled rat FSH (rFSH, 2.5 µg; 3×10^5 c.p.m./100 µl) in 100 µl FSH assay buffer consisting of 0.01 M PBS (pH 7.4), 0.1% BSA and 5 mM magnesium chloride. The cells were washed twice with 2 ml FSH assay buffer, and the bound ¹²⁵I activities in the granulosa cell pellets were counted on a gamma counter. Nonspecific binding (NSB) was assessed in the presence of excess FSH (purified from porcine pituitary extract, 500 µg/tube).

¹²⁵I-rLH binding assay

Aliquots (100 µl) of granulosa cells were incubated for 18 h at 37°C with a single dose of ¹²⁵I-labeled rat LH (rLH, 2.5 µg; 2×10^5 c.p.m./100 µl) in 100 µl of LH assay buffer (0.01 M PBS containing 0.1% BSA, pH 7.4). After incubation, the cells were washed twice with 2 ml LH assay buffer and counted on a gamma counter. NSB was assessed in the presence of excess chorionic hormone (hCG) (100 IU/tube).

The NSB was less than 1% of the added radioactivity in both the FSH and LH receptor binding assays. The amount of specific binding to rFSH and rLH was expressed as pg/2.5 × 10⁵ cells.

Aromatase activity assay

The aromatase activity assay was performed as previously described (Thatcher *et al.* 1991). Aliquots (100 µl) of

granulosa cells were incubated with [^3H]testosterone (0.5 μCi) in 4 ml Medium 199 for 4 h at 37°C in a 95% O_2 /5% CO_2 , humidified environment. The aromatase activity was measured as the stereospecific cleavage of ^3H from the radiolabeled testosterone and the consequent release of $^3\text{H}_2\text{O}$ into the medium; activity was expressed as d.p.m./ 2.5×10^5 cells.

Estradiol concentration in the follicular fluid

The estradiol content in the follicular fluid was measured by radioimmunoassay, as previously described (Kanai and Shimizu 1986). Briefly, the estradiol in the follicular fluid (200 μl) was extracted with 2.5 ml benzene. The minimum detection level of the assay was 1.0 pg/ml. The intra-assay coefficient of variation was 8.2%. The estradiol content in the follicular fluid was expressed as pg/ml per follicle.

Culture of follicles

Follicles at different developmental stages were dissected from the ovaries collected at 48 h after the PMSG injections in both groups. Culture of the follicles was performed as previously described (Chun *et al.* 1994). The follicles from three animals were pooled and cultured in glass tubes containing 1 ml Eagle's minimal essential medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin, streptomycin, L-glutamine and 0.1% BSA. Thirty early antral, 15 antral and 5 preovulatory follicles were cultured in each glass tube. The cultures were maintained for 6, 12, 18 and 24 h at 37°C and 95% O_2 /5% CO_2 . At each time point, the granulosa cells were isolated from the follicle, and a portion of the cells underwent Hoechst staining; the remainder were snap-frozen and stored at -80°C until DNA isolation.

Hoechst staining, DNA isolation and fragmentation analysis

Granulosa cells from follicles at different developmental stages were fixed in 1% glutaraldehyde overnight and stained with 1 mM Hoechst 33342 (Sigma) for 1 min. The samples were examined by fluorescence microscopy with an ultraviolet light filter. Calculation of the percentage of apoptotic granulosa cells, including shrunken cells, was based on a count of 200 cells.

Cellular DNA was extracted from the granulosa cells of preovulatory follicles and analyzed as previously described (Chun *et al.* 1994). Aliquots containing 500 ng DNA were labeled at the 3'-ends with [α - ^{32}P]dideoxy-ATP (25 μCi , 6000 Ci/mmol; Amersham Pharmacia Biotech, UK), using terminal transferase (Nippon Gene, Tokyo, Japan). Equal amounts of the labeled DNA samples (250 ng/lane) were fractionated by electrophoresis through 2% agarose gels. The gels were dried in a gel dryer and exposed on an imaging plate (Fuji Film Co. Tokyo, Japan). After exposure, the portion of each lane corresponding to DNA smaller than 15 kb was counted with a BAS-5000 computer-based

imaging system (Fuji Film) for quantitation of the degree of apoptotic DNA fragmentation. All data are expressed as the percentage of the control value at 0 h of culture.

Preparation of deletion-mutated cDNA competitors for competitive reverse transcription-polymerase chain reaction (RT-PCR) analysis

Partial fragments of the sequences encoding Bcl-2, Bax, and β -actin were obtained by RT-PCR with the following primers: *bcl-2* sense, 5'-CTG GGG ATG ACT TCT CTC G-3', and antisense, 5'-GGA GAA ATC AAA CAG AGG TC-3'; *bax* sense, 5'-AGA CAC CTG AGC TGA CCT TG-3', and antisense, 5'-GTC CCG AAG TAG GAG AGG AG-3'; *β -actin* sense, 5'-CAA CCT CCT TGC AGC TCC TC-3', and antisense, 5'-GAC CAG AGG CAT ACA GGG AC-3'. These fragments were blunt-ended, phosphorylated at their 5'-ends and inserted into the EcoRV site of pBlue-script KS (+) (Stratagene, La Jolla, CA, USA). The resultant *bcl-2* plasmid was digested with BstXI and HincII, and the *bax* plasmid was digested with AvaI to create the deletion mutations. The single-stranded ends were blunted with T4-DNA polymerase, and the plasmids were self-ligated. The mutated competitors were amplified by PCR, using the primers described above, and resolved by PAGE. The amplified cDNA fragments were recovered, quantified and used as internal standards for quantitative competitive RT-PCR.

Competitive RT-PCR

Total RNAs (3.0 μg) extracted from granulosa cells were reverse-transcribed with random primers, and the resultant cDNA mixtures were amplified by PCR, using the same primers described above in the presence of various amounts of the mutant competitor cDNAs and a trace amount of [α - ^{32}P]dCTP to permit quantification of the PCR products. The denaturation, annealing and polymerization cycles were performed as 58°C for 1 min, 65°C for 1 min and 58°C for 1 min respectively. The cycles were repeated 32, 30 and 28 times for amplification of the *bcl-2*, *bax* and *β -actin* sequences, respectively. The final incubation was continued at 72°C for 8.5 min to complete the polymerization. Native *bcl-2*, *bax*, and *β -actin* cDNAs produced 334, 378, and 490 bp fragments, respectively, whereas the cDNA for the deletion mutant competitors of *bcl-2*, *bax*, and *β -actin* generated 243, 263 and 377 bp fragments (target PCR product) respectively. The PCR products were fractionated by electrophoresis on 5% polyacrylamide gels. The gels were dried and analyzed by a BAS 5000 computer-based imaging system. The level of *β -actin* mRNA in rat follicle cells has been found to be independent of the follicle status and size (Tilly *et al.* 1992b), and *β -actin* expression is not affected by growth factors or gonadotropins (Hooley *et al.* 1979, Weiner and Dias 1993). Therefore, the quantitation of the *bcl-2* and *bax* PCR products was normalized to that of the *β -actin* PCR product.

Data analysis

All results were expressed as means $\pm$ standard error of the mean (S.E.M.). The significance of the differences in the amounts of FSH-R and LH-R, the aromatase activities in granulosa cells, the concentrations of estradiol in follicular fluid and the degrees of apoptotic DNA fragmentation at different developmental stages between the control and heat stress groups were analyzed by Student's *t*-test. The statistical differences in the percentages of apoptotic granulosa cells were analyzed by the arcsin test.

Results

Effects of heat stress on the number of granulosa cells, the aromatase activity and the estradiol concentration in follicular fluid before and after PMSG treatment

The number of granulosa cells obtained from follicles at different developmental stages increased as the follicle grew and did not differ significantly between the control and the heat stress groups before or after PMSG treatment (Fig. 1A and B). The aromatase activity in granulosa cells remained unchanged in preantral, early antral and antral follicles before PMSG treatment (Fig. 2A). By contrast, after PMSG treatment, the aromatase activity increased in granulosa cells from early antral, antral and preovulatory follicles in both the heat stress and control groups; however, the aromatase activity was lower in the heat stress group than that in the control group for all developmental stages (Fig. 2B). The estradiol concentration in the follicular fluid was low (baseline levels) in preantral, early antral and antral follicles before PMSG treatment (Fig. 3A). After PMSG treatment, the follicular estradiol concentration increased, particularly in antral and preovulatory follicles, in both groups, but the levels were lower in the heat stress group than those in the control group (Fig. 3B).

Effects of heat stress on FSH-R and LH-R in granulosa cells before and after PMSG treatment

The amount of FSH-R in granulosa cells before PMSG treatment increased as the follicles grew; a significantly lower amount of FSH-R in the heat stress group than in the control group was found only in the granulosa cells from antral follicles (Fig. 4A). After PMSG treatment, the amount of FSH-R in granulosa cells increased markedly in follicles at all developmental stages in both groups. However, compared with the controls, the heat stress group had significantly lower amounts of FSH-R in cells from early antral, antral and preovulatory follicles (Fig. 4B). The amount of LH-R in the granulosa cells remained unchanged in preantral, early antral and antral follicles in both groups before PMSG treatment (Fig. 5A). After PMSG treatment, the amount of LH-R increased markedly in the antral and preovulatory, but not early antral,

Figure 1 Effect of heat stress on the number of granulosa cells in follicles at different developmental stages harvested from immature female rat ovaries before (A) or after (B) PMSG treatment. Heat stress did not affect the number of granulosa cells before or after PMSG treatment. The data are presented as means $\pm$ S.E.M. of four independent experiments. PA, preantral; EA, early antral; A, antral; PF, preovulatory. The open and solid bars indicate the control and heat-stress groups respectively.

follicles of the control group; however, in the heat stress group, the levels of LH-R were significantly lower in the antral and preovulatory follicles than those in the controls (Fig. 5B).

Susceptibility to apoptosis of granulosa cells from heat-stressed follicles

Follicles at different developmental stages were obtained from ovaries at 48 h after the injection of PMSG and were cultured for 0, 6, 12, 18 and 24 h under serum-free conditions. Hoechst 33342 staining was used to examine the nuclear morphology of the granulosa cells from the follicles (Fig. 6A). Cells with small pyknotic nuclei and highly condensed chromatin were considered apoptotic. The percentage of apoptotic granulosa cells from early antral, antral and preovulatory follicles at 0 h of culture

Figure 2 Effect of heat stress on aromatase activity in granulosa cells of follicles at different developmental stages harvested from immature female rat ovaries before (A) or after (B) PMSG treatment. (A) Heat stress did not suppress aromatase activity in granulosa cells before PMSG treatment. (B) Heat stress suppressed aromatase activity in granulosa cells after PMSG treatment. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$). PA, preantral; EA, early antral; A, antral; PF, preovulatory. The open and solid bars indicate the control and heat-stress groups respectively.

did not differ significantly between the heat stress and control groups (Fig. 6A–C). The percentage of apoptotic cells increased markedly in the control group by 18 and 24 h in serum-free culture. In contrast, the percentage of apoptotic cells in the heat stress group increased in a time-dependent manner in follicles at each developmental stage.

The analysis of DNA fragmentation in granulosa cells from early antral, antral and preovulatory follicles cultured in serum-free conditions exhibited the typical apoptotic DNA degradation pattern of internucleosomal fragments of 185 bp multiples (Fig. 7A–C). The quantifications of the low-molecular-weight DNA content of early antral, antral and preovulatory follicles were consistent with the Hoechst stain observations and demonstrated a significant increase in apoptosis at 12 and 24 h of culture in the

Figure 3 Effect of heat stress on estradiol levels in the follicular fluid from follicles at different developmental stages harvested from immature female rat ovaries before (A) or after (B) PMSG treatment. (A) The estradiol in the follicular fluid before PMSG treatment was at baseline levels. Heat stress did not affect estradiol production before PMSG treatment. (B) Heat stress suppressed the level of estradiol in follicular fluid after PMSG treatment. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$). PA, preantral; EA, early antral; A, antral; PF, preovulatory. The open and solid bars indicate the control and heat-stress groups respectively.

antral and preovulatory follicles of the heat stress group compared with the control group (Fig. 7D–F).

The ratio of *bax* expression to *bcl-2* expression in granulosa cells

The level of *bcl-2* mRNA in granulosa cells from antral follicles was similar in both groups at 0 h of culture and was decreased in both groups at 12 h of culture (Fig. 8A). The level of *bax* mRNA did not differ significantly between the two groups at 0 h of culture (Fig. 8B), but the level in the heat stress group was significantly increased at 12 h compared with that at 0 h of culture. The ratio of the *bax* mRNA expression level to that of *bcl-2* was similar in the two groups at 0 h of culture, but by 12 h the ratio was significantly increased in the heat stress group compared with the control (Fig. 8C).

Figure 4 Effect of heat stress on FSH-R levels on granulosa cells of follicles at different developmental stages harvested from immature female rat ovaries before (A) or after (B) PMSG treatment. (A) Before PMSG treatment, heat stress suppressed FSH-R levels only on the granulosa cells of antral follicles. (B) After PMSG treatment, heat stress suppressed FSH-R levels on granulosa cells. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$). PA, preantral; EA, early antral; A, antral; PF, preovulatory. The open and solid bars indicate the control and heat-stress groups respectively.

Discussion

The results of the present study provide evidence that heat stress inhibits FSH-R expression in granulosa cells and results in low estrogenic activity in the follicles, that decreased FSH-R expression is associated with enhanced susceptibility to apoptosis in granulosa cells, and that the *bcl-2/bax* system may be not associated with heat-stress-induced apoptosis in granulosa cells.

In the present study, heat stress suppressed the aromatase activity in granulosa cells and resulted in a low capacity for estradiol production in the follicles. Several stress-associated factors, including corticoid and prolactin, affect aromatase activity. Prolactin levels were increased when animals were acutely exposed to high environmental temperatures (Hooley *et al.* 1979), thus inhibiting aromatase activity in rat granulosa cells (Tsai-Morris *et al.* 1983, Krasnow *et al.* 1990). In addition,

Figure 5 Effect of heat stress on LH-R levels on granulosa cells of follicles at different developmental stages harvested from immature female rat ovaries before (A) or after (B) PMSG treatment. (A) Before PMSG treatment, heat stress did not suppress LH-R levels in granulosa cells. (B) After PMSG treatment, heat stress suppressed LH-R levels in granulosa cells of antral and preovulatory follicles. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$). PA, preantral; EA, early antral; A, antral; PF, preovulatory. The open and solid bars indicate the control and heat-stress groups respectively.

glucocorticoids selectively inhibited FSH-induced aromatase activity in rat granulosa cells (Hsueh and Erickson 1978). Furthermore, in the present study, the inhibition of aromatase activity by heat stress may have resulted from increases in prolactin and glucocorticoid levels. The steroidogenic CYP19 gene encoding the aromatase cytochrome P450 enzyme is induced in granulosa cells from rat preovulatory follicles *in vivo* (Hickey *et al.* 1988) and *in vitro* (Hickey *et al.* 1990, Krasnow *et al.* 1990). Aromatase mRNA can be superinduced in cells differentiated by exposure to LH and cycloheximide, suggesting that the steady-state levels of mRNA involve not only transcriptional control but also mRNA stability (Fitzpatrick and Richards 1991). The rat aromatase promoter has a TATA motif and a single transcriptional initiation site (Fitzpatrick and Richards 1991). Thus, heat stress may affect transcriptional regulation and control of

Figure 6 Detection of apoptosis in granulosa cells of cultured early antral (A), antral (B) and preovulatory (C) follicles with Hoechst 33342 staining. Follicles at different developmental stages were cultured for 0, 6, 12, 18 and 24 h in serum-free medium. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$) compared with the controls. The solid and open circles indicate the control and heat-stress groups respectively.

the initiation site, as well as stability of aromatase mRNA, in granulosa cells.

Our findings indicate that heat stress suppresses LH-R expression in granulosa cells of antral and preovulatory follicles after PMSG treatment. As LH-R expression in granulosa cells is induced by estradiol, heat-stress-induced decreases in the estradiol concentration in the follicular fluid may result in the inhibition of LH-R expression in the granulosa cells. Interestingly, in the present study, heat stress induced a decrease in the estradiol concentration in the follicular fluid of early antral follicles after PMSG treatment, but did not suppress LH-R expression in the granulosa cells. LH/hCG receptors are expressed

predominantly on the thecal cells of small antral follicles, whereas LH/hCG receptors are expressed on both granulosa and thecal cells in large antral follicles (Camp *et al.* 1991, Peng *et al.* 1991, Teerds and Dorrington 1995). Our findings suggest that heat stress may inhibit LH-R expression in thecal cells, rather than in granulosa cells, of follicles at the early antral stage.

FSH receptors are confined to the granulosa cells of healthy, developing follicles (Camp *et al.* 1991). Estradiol contributes to the maintenance and expression of both LH-R and FSH-R in granulosa cells (Hsueh *et al.* 1994, Bodensteiner *et al.* 1995). In the present study, we found that, although the concentration of estradiol in the follicular fluid was already at baseline levels before PMSG treatment, heat stress nonetheless inhibited FSH-R expression in the granulosa cells of antral follicles. This result suggests that FSH-R expression in the granulosa cells is also regulated by estradiol-independent mechanisms.

The administration of PMSG results in a marked increase of FSH-R mRNA expression and FSH-binding sites (Nakamura *et al.* 1991, LaPolt *et al.* 1992). FSH-R expression in granulosa cells is also induced by locally produced intraovarian growth factors, such as transforming growth factor-beta (TGF- β) (Tilly *et al.* 1992a, Gitay-Goren *et al.* 1993, Dunkel *et al.* 1994) and insulin-like growth factor-I (IGF-I) (Zhou *et al.* 1997). Our study indicates that heat stress suppresses FSH-R expression in granulosa cells of follicles at the early antral, antral and preovulatory stages after PMSG treatment. This finding suggests that the increase in atresia caused by heat stress (Shimizu *et al.* 2000) may be induced by the inhibition of FSH-R expression in granulosa cells.

The lack of follicular survival factors such as FSH and estradiol leads to granulosa cell apoptosis characterized by DNA fragmentation and follicular somatic cell condensation. In the present study, granulosa cells of follicles harvested from heat-stressed, immature, female rats after PMSG treatment rapidly underwent DNA fragmentation when cultured under serum-free conditions, suggesting that heat-stressed follicles are highly susceptible to apoptosis. Thus, we hypothesized that apoptosis-related genes might already be expressed at 0 h of culture time in the granulosa cells of heat-stressed follicles.

One key group of intracellular factors regulating apoptosis is the Bcl-2 family of proteins (Adams and Cory 1998). The members of this family can be subdivided into antiapoptotic proteins (such as Bcl-2 and Bcl-xL) and proapoptotic proteins (such as Bax and BAD). It has been proposed that anti- and proapoptotic proteins regulate cell death by binding to each other and forming heterodimers (Oltvai *et al.* 1993, Yang *et al.* 1995). According to this model, a delicate balance between anti- and proapoptotic Bcl-2 family members exists in each cell, and the relative concentrations of these two groups of proteins determine whether the cell survives or undergoes apoptosis. To investigate our hypothesis, we examined the expression levels of *bcl-2* and *bax* mRNAs in granulosa cells, using

Figure 7 Detection of DNA fragmentation in granulosa cells of cultured early antral (A and D), antral (B and E) and preovulatory (C and F) follicles. Follicles at different developmental stages were harvested from control (C) or heat-stressed (H) ovaries and cultured for 0, 12 and 24 h in serum-free medium. After incubation, granulosa cell DNA was extracted, labeled at the 3'-ends with [32 P]dideoxy-ATP, and fractionated through 2% agarose gels (500 ng/lane). DNA fragmentation was then visualized by autoradiography (A–C). The portions of each lane corresponding to DNA smaller than 15 kb were counted by an image-analysis system for quantitation of the degree of apoptotic DNA fragmentation. All data are expressed as the percentage of the control value at 0 h of culture. The data are means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$) compared with the controls. The open and solid bars indicate the control and heat-stress groups respectively.

quantitative RT-PCR. Our findings indicate that the levels of *bcl-2* and *bax* mRNA, and the ratio of *bax* to *bcl-2* mRNA, were similar in both treatment groups at 0 h of culture. These observations suggest that *in vivo* heat stress did not affect the *bcl-2/bax* apoptotic signaling pathway at the beginning of granulosa cell apoptosis *in vitro*. However, the expression of *bax* mRNA in granulosa cells of follicles from heat-stressed animals increased significantly after the 12-h culture period. This result suggests that the amount of *bax* present in granulosa cells that maintain a constitutive level of *bcl-2* expression may be linked to the induction of granulosa cell apoptosis and follicular atresia mediated by heat-stress. It is known that another apoptotic signaling cascade, the Fas/Fas ligand (FasL) system, can lead to granulosa cell apoptosis (Quirk *et al.* 1995,

Hakuno *et al.* 1996, Kim *et al.* 1998). Thus, it is possible that heat stress stimulates the Fas/FasL cascade in the granulosa cells of growing follicles.

In conclusion, heat stress inhibits the FSH-R signaling pathway in the granulosa cells of growing follicles, causing decreased estrogen levels, which are known to produce increased follicular atresia. Thus, the regulators of FSH-R expression may be potential targets for therapeutic intervention for low summer fertility syndrome, as a substitute for therapy with estrogenic compounds.

Acknowledgements

We thank Dr Miho Hirabayashi for her helpful suggestions on RT-PCR analysis, NIDDK's National Hormone and Pituitary

Figure 8 Effects of heat stress on expression of *bcl-2* (A) and *bax* (B) mRNA and the ratio of *bcl-2*/*bax* (C) in granulosa cells. Total RNAs (3.0 µg) were reverse-transcribed with random primers, and the resultant cDNA mixtures were amplified by PCR in the presence of various amounts of deletion mutant competitor cDNAs and a trace amount of [α - 32 P]dCTP to quantify the PCR products. The PCR products were size fractionated by electrophoresis on 5% polyacrylamide gels. The data are presented as means $\pm$ S.E.M. of four independent experiments. Asterisks denote significantly different values ($P < 0.05$) compared with the controls. The open and solid bars indicate the control and heat-stress groups respectively.

Program and Dr A F Parlow for the purified rat FSH and LH. This work was partly supported by a Grant-in-Aid for Exploratory Research (14656098) from the Japan Society for the Promotion of Science. The authors declare that there is no conflict of interest that would affect prejudice the impartiality of this scientific work.

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Received 30 September 2004

First decision 18 November 2004

Accepted 21 December 2004