Haem oxygenase augments porcine granulosa cell apoptosis *in vitro*

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Abstract

Haem oxygenases produce carbon monoxide, which, like nitric oxide, is a gaseous messenger molecule that is one of several important survival factors in ovarian follicles. However, little is known about the expression and possible functions of these enzymes in granulosa cells. The purpose of this study was to investigate the expression and possible role of haem oxygenases in porcine granulosa cells (PGCs). We obtained frozen sections of porcine ovaries and PGCs from ovarian follicles of various sizes by needle aspiration, and examined the expression of haem oxygenase-1 (HO-1; inducible type) and HO-2 (constitutive type) in PGCs by immunohistochemistry, RT-PCR, western blotting and flow cytometry. Both types of haem oxygenase were identified in PGCs throughout follicular development, but HO-1 was expressed primarily in granulosa cells in atretic follicles. We also investigated the effect of haem oxygenases on apoptosis of granulosa cells (flow cytometry to detect subdiploid DNA fluorescence) and on expression of Fas ligand (quantitative analysis of

western blotting and flow cytometry). In tightly bound PGCs, the mean proportion of apoptotic cells treated with 1 μ M haemin (a haem oxygenase substrate) was approximately 1.7-fold greater than that in untreated controls, and zinc protoporphyrin IX (ZnPP IX; a haem oxygenase inhibitor) completely inhibited the increase in apoptosis induced by haemin in 24-h culture. Conversely, in weakly associated PGCs, the proportion of apoptotic cells was not altered by haemin. The quantity of Fas ligand protein was increased in a dose-dependent manner in tightly bound PGCs treated with haemin compared with controls, and the haemin-induced increase in Fas ligand protein was inhibited by ZnPP IX. Thus we identified inducible HO-1 and constitutive HO-2 in PGCs throughout follicular development, and we conclude that products of reactions catalysed by haem oxygenases are likely to be important autocrine/paracrine factors that regulate apoptosis in PGCs.

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Introduction

Carbon monoxide (CO) is a gaseous messenger molecule that has diverse cellular functions (Otterbein & Choi 2000). It has a role similar to that of nitric oxide (NO) in some organs: it is considered to be a neurotransmitter like NO in the central nervous system (Costa *et al.* 1996, Kim & Rivier 2000), and both CO and NO stimulate the action of soluble guanylate cyclase, thereby promoting smooth muscle relaxation and vasodilatation in the cardiovascular system (Villamor *et al.* 2000). CO is produced endogenously, mainly from the degeneration of haem by haem oxygenases (Maines 1988).

Haem oxygenases are the rate-limiting enzymes in haem degradation, catalysing the cleavage of the haem ring to form free iron, carbon monoxide and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase, and free iron is promptly sequestered into ferritin. Three distinct variants of haem oxygenases have been identified in humans. HO-1 is an inducible enzyme, the synthesis of which is elicited by numerous stimulants such as cytokines, heat shock, heavy metals,

hypoxia, endotoxins and oxidants that induce inflammatory damage (Yoshida *et al.* 1988, Eyssen-Hernandez *et al.* 1996, Carraway *et al.* 1998, Otterbein & Choi 2000, Panchenko *et al.* 2000, Stuhlmeier 2000). In contrast, HO-2 is constitutively expressed and is concentrated mostly in the brain and testes (McCoubrey *et al.* 1992, Otterbein & Choi 2000). HO-3 is also localized primarily in the brain, although its enzymatic activity is lower than that of the other isoenzymes (McCoubrey *et al.* 1997). HO-1 reduces vasoconstriction and inhibits cell proliferation during vascular injury in pig arteries (Duckers *et al.* 2001). HO-2 protein is highly expressed in large and small blood vessels of newborn pig cerebrum and its product, CO, contributes to vasodilatation (Leffler *et al.* 1999). Betamethasone upregulates HO-2 protein in endothelial cells of the cerebral microvascular of newborn pigs (Parfenova *et al.* 2001). Thus the roles of HO-1 and HO-2 in various physiological phenomena have been investigated in porcine models.

In granulosa cells, the NO exerts an inhibitory effect through a cGMP-dependent pathway (Chun *et al.* 1995). Some investigators have also found that the NO/nitric

oxide synthase (NOS) system inhibits steroid production and P450 aromatase activity in human luteal granulosa cells and in porcine granulosa cells (PGCs) (Van Voorhis *et al.* 1994, Masuda *et al.* 1997). As CO and NO have similar cellular functions in several organ systems, including the central nervous system (Kim & Rivier 2000) and the cardiovascular system (Villamor *et al.* 2000), the CO/haem oxygenase system also may be involved in the follicular development of the ovaries.

During follicular development, the majority of ovarian follicles are destined to undergo atresia, which is known to occur via apoptosis throughout female life (Tilly *et al.* 1991, Kaipai & Hsueh 1997). Apoptosis of follicular cells occurs at all stages of follicle development, and is regulated by several intraovarian factors, including tumour necrosis factor (TNF)- α , interleukin (IL)-6, and gonadotrophin releasing hormone (Gorospe *et al.* 1992, Billig *et al.* 1993, Santana *et al.* 1995, Kaipai *et al.* 1996). In addition, overexpression of Bcl-2 in follicular cells inhibits cell apoptosis and increases the frequency of germ cell tumours in ageing animals (Hsu *et al.* 1996). Therefore, follicular cell apoptosis is also important for normal ovarian development.

Haem oxygenase has various cellular functions in endocrine organs. In rat cultured pancreatic islets, it provides a protective role against the actions of IL-1 β that induce cell apoptosis and inhibit insulin release (Ye & Laychock 1998). In rat adrenals, treatment with tin protoporphyrin IX (SnPP IX), a haem oxygenase inhibitor, attenuates serum corticosterone concentrations by inhibiting the activities of both microsomal 21 α -hydroxylase and mitochondrial 11 β -hydroxylase (Maines & Trakshel 1992). In rat testes, SnPP IX also inhibits the activities of cytochrome P450 and microsomal 17 α -hydroxylase (Maines & Trakshel 1992). Furthermore, because cadmium chloride-induced HO-1 augmented the rate of apoptosis of premeiotic germ cells, the CO/haem oxygenase system was considered to regulate homeostasis of spermatogenesis under conditions of stress (Ozawa *et al.* 2002). In ovaries, haem oxygenase may have a regulatory role during oogenesis. However, the possibility that haem oxygenase might stimulate steroidogenesis in rat ovary was reported only recently (Alexandrea & Lawson 2003), and little is known about the possible functions of haem oxygenases in the female reproductive organ.

In the present study, we investigated the expression of inducible type (HO-1) and constitutive type (HO-2) haem oxygenases in PGCs by means of immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR), western blotting and flow cytometry. Furthermore, we examined these haem oxygenases separately in two subpopulations of PGCs, weakly associated and tightly bound, which are distinguished by size and have different functional characteristics (Marrone & Crissman 1988, Howard & Ford 1994, Duda *et al.* 1999). Subsequently, we investigated the effects of haem

oxygenase substrate and haem oxygenase inhibitor on granulosa cell apoptosis, using flow cytometry to measure subdiploid DNA fluorescence, and on the expression of Fas ligand, using quantitative analysis of western blotting and flow cytometry immunocytochemistry. Our results demonstrated that both haem oxygenases, inducible and constitutive, were expressed throughout follicular development and that haem oxygenase products augmented the expression of Fas ligand protein and apoptosis in PGCs.

Materials and Methods

Immunohistochemistry

Serial frozen sections (8 μ m thick) of porcine ovary samples obtained from 4–6-month-old Yorkshire pigs at a local slaughterhouse were mounted onto silane-coated slides, and then fixed in acetone at 4 °C for 15 min. Thereafter, sections were treated with 3% hydrogen peroxide in absolute methanol for 10 min in order to block endogenous peroxidase activity. After a washing with PBS, pH 7.2, the sections were preincubated for 30 min at room temperature with Dulbecco's PBS (D-PBS; Takara, Tokyo, Japan) containing 5% normal goat serum, to prevent non-specific binding of the antibodies. They were then incubated with anti-HO-1 (1:100) or anti-HO-2 (1:200) polyclonal antibody (Stressgen, Victoria, Canada) for 1 h at room temperature. After washing with PBS, the sections were incubated for 30 min at room temperature with Histofine simple stain PO (Nichirei Co., Tokyo, Japan). The histochemical reaction method for detecting peroxidase activity was carried out as described in a previous report (Harada *et al.* 2002). The immunostained sections were counterstained with Mayer's haematoxylin. The specificity of these antibodies in pig tissues, and their lack of cross-reactivity, have already been reported (Parfenova *et al.* 2001) and we reconfirmed their specificities by band size in western blotting analysis. Negative controls were prepared by replacing the primary antibody with an appropriately diluted normal rabbit serum.

Cell collection

Porcine granulosa cells were collected from 4–6-month-old Yorkshire pigs and cultured as described previously (Masuda *et al.* 1997). PGCs were isolated from different stages of follicles, classified as small (<2 mm), medium (3–5 mm), or large (>6 mm), by needle aspiration. For protein and mRNA extraction, cells were washed twice with D-PBS, and then stored at –80 °C until required for use. For immunocytochemistry using flow cytometric analysis, cells were incubated with 2 mg/ml collagenase type 1 (Sigma) and 100 μ g/ml deoxyribonuclease (DNase) (Sigma)/D-PBS containing 0.133 mg/ml calcium dichloride for 20 min at 37 °C for dissociation, and washed twice with D-PBS before the next procedure.

To determine the effect of haem oxygenase on granulosa cell apoptosis, PGCs were isolated from medium follicles (3–5 mm) by the needle aspiration method. After enzymatic dissociation as described above, the cells were washed and then suspended in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Inc.) containing 10% FCS (Nichirei Co.) and 1% antibiotic–antimycotic solution, containing 10 000 U/ml penicillin G, 10 000 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B (Life Technologies Inc.). Cells were seeded on 10 cm culture dishes and preincubated for 48 h at 37 °C in a humidified 95% air–5% CO₂ incubator before the examination. On the day of experiment, the medium was changed and the cells were incubated with various compounds in serum-free, phenol-red-free DMEM.

Separation of two subpopulations of PGCs

Porcine granulosa cells were enzymatically dissociated as described above, and then separated by filtration on a 0.8 µm nylon mesh filter. Weakly associated PGCs passed through the filter, but whole PGCs did not. Both groups of cells were fixed with 4% paraformaldehyde/D-PBS and were then used to set the gate for flow cytometry experiments, separating the two subpopulations of PGCs: the two subpopulations have differential channels of forward scatter, which reflects cell size (Marrone & Crissman 1988). A Beckton Dickinson fluorescence-activated cell sorter (FACS) Calibur machine was used for analysis, after calibration with CaliBrite beads.

RNA isolation and RT-PCR

Total RNA was isolated from PGCs using RNeasy mini kits (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The quantity of extracted total RNA was determined by spectrophotometry at 260 and 280 nm. Complementary DNA was prepared from 1 µg DNase-treated RNA using the SuperScript First-Strand Synthesis System (Life Technologies Inc.), and one-tenth of the mixture was treated with RT and used as the template for PCR. The primer sequences for porcine HO-1 were designed according to published cDNA sequences. The forward 5'-CAGCCCAACAGCATGCC CCAG-3' (87–107) and the reverse 5'-CGTAGCGC TTGGTGGCCTGCG-3' (417–397) primers were used to amplify a 331 bp fragment. The primer sequences for porcine HO-2 were designed according to the methods of Parfenova *et al.* (2001). The forward 5'-GGACA AYGCSAGCAGTTCAAGCAG-3' and the reverse 5'-CGCAYRTCTCCTTTCCCATCGTG-3' primers were used to amplify a 213 bp fragment. For the detection of porcine HO-1 and HO-2 mRNA in PGCs, PCR amplification was carried out using a PCR core kit (Roche). Samples were amplified in a 50 µl PCR solution

(10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 IU Taq polymerase) with 30 cycles of the following sequential steps: denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s in a Perkin-Elmer GeneAmp 2400 Thermal Cycler. The PCR products were subjected to 2.0% agarose gel (Takara) electrophoresis and visualized by ethidium bromide staining. The sequences of the PCR products were determined using an automated ABI Prism 310 sequencer (Perkin-Elmer Co.) and compared with those of HO-1 and HO-2 to confirm their identity. PCR amplification of samples without prior RT was performed as a negative control.

Western blotting analysis of haem oxygenases and Fas ligand

Porcine granulosa cells were obtained from different stages of follicles and cultured with haemin, a haem oxygenase substrate (Affiniti Research Product Ltd, Exeter, Devon, UK) or zinc protoporphyrin IX (ZnPP IX), a haem oxygenase inhibitor (Oxis, Portland, OR, USA), or both, for 24 h. They were then lysed with lysis buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 1% Nodidet P-40, 0.1% deoxycholic acid, 10 µM phenyl methylsulphonyl fluoride, 0.5 mU/ml apoprotinin, 1 µM leupeptin, 1 µM pepstatin) to analyse the expression of haem oxygenases and Fas ligand protein. Protein concentration was measured with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) using BSA as the standard. Each sample contained equivalent amounts of protein (40 µg for experiments detecting expression of haem oxygenases and 15 µg for experiments detecting Fas ligand), which were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Atto, Tokyo, Japan). After blocking with Block Ace (Dainippon, Osaka, Japan) containing 10% FCS to prevent non-specific binding of the antibodies, the membranes were incubated with anti-HO-1 (1:1000) or anti-HO-2 (1:2000) polyclonal antibody (Stressgen), or with anti-Fas-ligand (1:1000) rabbit polyclonal antibody (Stressgen) for 1.5 h at room temperature. After a washing with buffer, the membranes were sequentially incubated with horseradish-peroxidase-conjugated anti-rabbit IgG antibody (1:5000) (Amersham). The bound antibodies were detected by enhanced chemiluminescence (Amersham) in accordance with the recommended procedure. Quantification was performed by computerized optimal densitometric scanning of images (National Institutes of Health image program), and then normalized using β-actin protein as an internal standard.

Immunocytochemical analysis of haem oxygenases and Fas ligand by flow cytometry

To detect the expression of haem oxygenases, PGCs obtained from different stages of follicles were dissociated

enzymatically as described above. The cells were fixed in 4% paraformaldehyde/D-PBS for 30 min at room temperature and then incubated with permeabilizing solution (5% diethylene glycol and 1.5% formaldehyde/D-PBS) (Becton Dickinson) for 10 min at room temperature. For analysis of Fas ligand, PGCs were cultured with different concentrations of two compounds, haemin and ZnPP IX, for 24 h. All cells that remained adherent to the culture plates and PGCs that were floating in the culture medium were recovered and fixed in 4% paraformaldehyde/D-PBS for 30 min at room temperature. After three washes with cold D-PBS, cells were preincubated for 30 min at 4 °C in D-PBS containing 5% normal goat serum and 10% FCS to prevent non-specific binding of antibodies for both haem oxygenases and Fas ligand. Cells were subsequently incubated with anti-HO-1 (1:50) or anti-HO-2 (1:50) polyclonal antibody (Stressgen) and anti-Fas-ligand (1:50) rabbit polyclonal antibody (Stressgen) for 30 min at 4 °C. After two washes with cold D-PBS, the cells were incubated with fluorescein-isothiocyanate-conjugated anti-rabbit IgG (Southern Biotechnology Associate, Birmingham, AL, USA) for 30 min at 4 °C. The cells were centrifuged, resuspended in 1.5 ml 1% paraformaldehyde/D-PBS on ice, and analysed using a Beckton Dickinson FACS Calibur machine. At least 10 000 events were recorded separately. Negative controls were prepared by replacing the primary antibody with an appropriately diluted normal rabbit serum and control rabbit IgG for haem oxygenases and Fas ligand, respectively.

Analysis of DNA contents in granulosa cells by flow cytometry

Porcine granulosa cells were seeded in 10 cm dishes and preincubated for 48 h at 37 °C in a humidified 95% air–5% CO₂ incubator. After preincubation, the medium was changed and the cells were incubated with haemin or ZnPP IX, or both, in serum-free phenol-red-free DMEM for different times (4 h, 8 h, 16 h, 24 h) and at different concentrations (0.1, 1 µM). Floating cells and trypsinized cells, which were originally attached to the bottom of the culture dishes after each treatment, were collected and combined to ensure a complete recovery of the cell population. After washing with cold D-PBS, the cells were fixed in cold 70% ethanol, and then stored at –20 °C until required for use (more than 4 h). On the day of experiment, cells were centrifuged and supernatants were completely removed, and then cells were incubated in 200 µl phosphate–citrate buffer (0.2 M Na₂HPO₄ 192 µl and 0.1 M citric acid 8 µl) at room temperature for 30 min. After incubation, cells were stained for 30 min with 50 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) and 50 µg/ml ribonuclease A (Sigma)/D-PBS containing 0.1% Triton-X (Sigma). Cells were filtered through a 35 µm nylon mesh filter (Becton Dickinson) and then analysed by FACS. The percentage of sub-G1 phase

(apoptotic) cells was calculated by CELL QUEST software. At least 30 000 events were recorded in each specimen.

Statistical analysis

For immunohistochemical localization, sections from four independent porcine ovary samples were observed. All experiments were repeated at least three times to ensure reproducibility of the results, and the values were expressed as the means ± s.d. For comparison between unpaired groups, ANOVA was used. A *P* value less than 0.05 was considered statistically significant.

Results

Determination of haem oxygenase localization by immunohistochemistry

Immunostaining for HO-1 and HO-2 was observed in all porcine ovaries studied; representative photomicrographs are shown in Figure 1. HO-1 protein was weakly expressed in PGCs in healthy follicles (Fig. 1A). In contrast, granulosa cells were stained intensely with the anti-HO-1 antibody in atretic follicles (Fig. 1B), in which large numbers of loose, pyknotic granulosa cells were found (Chaffin & Stouffer 2000). Conversely, HO-2 protein was observed in granulosa cells in healthy follicles (Fig. 1C), whereas immunostaining for HO-2 was weak in granulosa cells in atretic follicles (Fig. 1D).

Detection of haem oxygenases mRNA by RT-PCR

Polymerase chain reaction amplification of PGC cDNA with the HO-1 and HO-2 primer sets generated the expected 331 bp and 213 bp fragments, respectively, from all PGC RNA samples (Fig. 2A, B), and the sequences of the products were identical to those of HO-1 and HO-2. Thus HO-1 and HO-2 mRNA was detected by RT-PCR in PGCs at all stages of follicular development.

Detection of haem oxygenase proteins by western blotting analysis

Immunoreactivity against HO-1 and HO-2 proteins was detected in PGCs extracted from small, medium and large follicles, by western blotting. The HO-1 antibody produced a 32 kDa protein band corresponding to the approximate molecular mass of porcine HO-1 protein, and we detected HO-1 in protein extracts from PGCs at all stages of follicular development (Fig. 2C). The HO-2 antibody produced a 36 kDa protein band corresponding to the approximate molecular mass of porcine HO-2 protein (Parfenova *et al.* 2001) and we also detected HO-2

Figure 1 Immunohistochemical localization of haem oxygenase (HO)-1 and -2 proteins in the porcine ovary. (A) Stained with anti-HO-1 polyclonal antibody in the healthy follicle. (B) Stained with anti-HO-1 polyclonal antibody in the atretic follicle. (C) Stained with anti-HO-2 polyclonal antibody in the healthy follicle. (D) Stained with anti-HO-2 polyclonal antibody in the atretic follicle. (E) Negative control (healthy follicle). (F) Negative control (atretic follicle). The immunostained sections were counterstained with Mayer's haematoxylin. Original magnification: $\times 400$.

in protein from PGCs at all stages of follicular development (Fig. 2D).

Determination of two subpopulations of PGCs by flow cytometry

A contour line analysis, forward scatter versus side scatter, was performed in whole PGCs and weakly associated PGCs. In the contour line of whole granulosa cells, containing both tightly bound and weakly associated PGCs, two peaks were identified (Fig. 3A). In contrast, in the contour line of weakly associated PGCs, only one peak

was determined, which corresponded to the left peak of the previous picture (Fig. 3B). Therefore, we were able to set the gate (Fig. 3C) for the investigation of each subpopulation of PGCs, tightly bound or weakly associated, in subsequent experiments.

Detection of haem oxygenases in two subpopulations of PGCs by flow cytometric analysis

Two subpopulations of PGCs, tightly bound and weakly associated, were analysed separately by setting gates on the basis of the findings of the previous experiment. The

Figure 2 (A, B) RT-PCR analysis of expression of mRNA for haem oxygenase isozymes in porcine granulosa cells (PGCs) at each stage of follicular development. (A) HO-1 specific primers generated the expected 331 bp fragment from all PGC RNA samples. (B) HO-2 specific primers generated the expected 213 bp fragment from all PGC RNA samples. (C, D) Western blotting analysis of haem oxygenase isozymes using HO-1 and HO-2 polyclonal antibodies against proteins extracted from PGCs in each stage of follicular development. (C) Anti-HO-1 antibody produced a protein band at 32 kDa (approximate molecular mass of porcine HO-1). (D) Anti-HO-2 antibody produced a protein band at 36 kDa (approximate molecular mass of porcine HO-2). MM, 100 bp ladder marker; SF, small follicles (< 2 mm); MF, medium follicles (3–5 mm); LF, large follicles (> 6 mm).

histograms of the number of PGCs stained with HO-1 antibody or normal rabbit serum (negative control) are shown in Figure 4A. Granulosa cells staining with HO-1 antibody had greater fluorescence intensity compared with negative controls, which is indicated in the histogram by a shift to the right. These results showed that HO-1 proteins were expressed in both tightly bound and weakly associated PGCs throughout follicular development. The histograms of PGCs stained with HO-2 antibody or normal rabbit serum (negative control) are shown in Figure 4B. HO-2 proteins were also expressed in both tightly bound and weakly associated PGCs throughout follicular development.

Effect of haem oxygenase on granulosa cell apoptosis by a sub-G1 phase population in DNA content analysis

We examined the effect of haemin on the number of PGCs using a WST-1 cell proliferation kit (Roche) before analysis for DNA content. The number of PGCs was significantly changed by treatment for 24 h with haemin in concentrations ranging from 0.1 μ M to 1 μ M, but was

not altered by culture with less than 0.01 μ M of this agent (data not shown). Culture with haemin at 0.1 μ M and 1 μ M was therefore considered to be suitable to examine the effect of haemin on granulosa cell apoptosis. We calculated the percentage of apoptotic PGCs treated with haemin to determine the effect of this compound on granulosa cell apoptosis. The two subpopulations of PGCs were analysed separately.

The effects of treatment of tightly bound PGCs with 1 μ M haemin at different times of culture are shown in Figure 5. The apoptotic degradation of DNA induced by haemin treatment is shown in representative DNA histograms of tightly bound PGCs treated with or without 1 μ M haemin at different culture times (Fig. 5A). At the beginning of treatment (0 h), the proportion of sub-G1 phase PGCs, which contain subdiploid amounts of DNA, was low. The proportion of sub-G1 cells increased gradually and in a similar way in both haemin-treated and haemin-untreated cells for the first 16 h, but the percentage of apoptotic cells was significantly greater at 24 h in haemin-treated cells. The results from three separate experiments are shown in Figure 5B. At the beginning of

Figure 3 Two subpopulations of porcine granulosa cells (PGCs) separated by flow cytometry. (A) Contour line analysis in whole granulosa cells, containing tightly bound and weakly associated PGCs. (B) Contour line analysis in weakly associated PGCs, which were passed through the 0.8 μ m filter. (C) Setting gates of both tightly bound and weakly associated PGCs on the picture of dot plot analysis. We used the gate to investigate each subpopulation of PGCs in subsequent experiments.

treatment, the mean percentage of apoptotic cells was low ($0.27 \pm 0.17\%$, mean $\pm$ S.D.), and reached approximately 10.0% at 24 h of culture in untreated tightly bound PGCs. However, in tightly bound PGCs treated with 1 μ M haemin, the mean percentage of apoptotic cells continued to increase after 16 h of culture and was significantly increased compared with controls at 24 h of culture ($16.5 \pm 3.4\%$ compared with $10.0 \pm 2.7\%$, $P < 0.05$).

Representative DNA histograms of weakly associated PGCs treated with or without 1 μ M haemin at different culture times are shown in Figure 6A. At the beginning of treatment (0 h), the proportion of sub-G1 phase GCs was low, but the percentage of apoptotic cells among haemin-treated and haemin-untreated weakly associated PGCs increased dramatically over 24 h of culture time. The percentage of apoptotic weakly associated PGCs was not

altered by treatment with 1 μ M haemin at any observed time point. The results from three separate cultures are shown in Figure 6B. The mean percentage of apoptotic cells was low at 0 h ($6.0 \pm 3.1\%$), and was significantly increased at 4 h of culture in serum-free conditions ($50.0 \pm 6.8\%$). The percentage of apoptotic cells in weakly associated PGCs in untreated control culture continued to increase and reached approximately 90.0% at 24 h culture. In weakly associated PGCs treated with 1 μ M haemin, the mean percentage of apoptotic cells increased in a manner similar to that in control cultures.

To confirm the effect of haemin on apoptosis in tightly bound PGCs and the inhibitory effect of ZnPP IX on haemin-induced apoptosis, we cultured the cells with haemin or haemin and ZnPP IX at different concentrations for 24 h. Representative DNA histograms of

Figure 4 (A) Immunocytochemistry analysis of HO-1 expression in porcine granulosa cells (PGCs) by flow cytometry. PGCs staining with HO-1 antibody had greater fluorescence intensity than negative controls, so the histogram of cells staining with HO-1 antibody was shifted to the right. These results indicate that HO-1 proteins were expressed in both tightly bound and weakly associated PGCs throughout follicular development. (B) Immunocytochemistry analysis of HO-2 expression in PGCs using flow cytometry. These results show that HO-2 proteins were expressed in both tightly bound and weakly associated PGCs throughout follicular development.

Figure 5 Effect of culture time and haemin, a haem oxygenase substrate, on apoptosis in tightly bound porcine granulosa cells (PGCs). (A) Representative DNA histograms in tightly bound PGCs treated with or without haemin at different culture times. (B) Mean percentage of apoptotic cells at different culture times in tightly bound PGCs. ○, Control (serum starvation only); ●, treated with haemin 1 μM. Data are means ± S.D. of three independent experiments. * $P < 0.05$ compared with control at the same culture time.

tightly bound PGCs are shown in Figure 7A. The proportion of sub-G1 phase cells in tightly bound PGCs was significantly increased by treatment with 1 μM haemin compared with control culture. The proportion of sub-G1 phase cells in tightly bound PGCs treated with 0.1 or 1 μM ZnPP IX in the presence of 1 μM haemin was decreased compared with cultures treated with 1 μM haemin. The results of four separate experiments in tightly bound PGCs are summarized in Figure 7B. The mean percentage of apoptotic cells was significantly increased by treatment with haemin: the rate of apoptosis among cells treated with 1 μM haemin was approximately 1.7-fold increased compared with that in untreated control cultures ($17.2 \pm 1.9\%$ compared with $9.8 \pm 1.9\%$, $P < 0.05$). Furthermore, compared with that in haemin-treated cultures, the mean percentage of apoptotic cells was

significantly and dose-dependently decreased by treatment with ZnPP IX in the presence of 1 μM haemin.

Effect of haem oxygenase on Fas ligand by quantitative analysis of western blotting

To investigate the correlation between haemin-induced apoptosis and expression of Fas ligand, we sought to determine the expression of Fas ligand in PGCs treated with haemin, using western blotting. The Fas ligand antibody produced a 38 kDa protein band identical to the approximate molecular mass of porcine Fas ligand protein. The quantity of Fas ligand protein was significantly increased ($P < 0.05$) by treatment with haemin, in a dose-dependent manner (Fig. 8A). The results of the densitometric analysis are shown in Figure 8B. The

Figure 6 Effect of culture time and haemin, a haem oxygenase substrate, on apoptosis in weakly associated porcine granulosa cells (PGCs). (A) Representative DNA histograms in weakly associated PGCs treated with or without haemin at different culture times. (B) Mean percentage of apoptotic cells at different culture times in weakly associated PGCs. ○, Control (serum starvation only); ●, treated with haemin 1 μ M. Data are means $\pm$ S.D. of three independent experiments.

quantity of Fas ligand protein from PGCs treated with 1 μ M haemin was approximately 1.5-fold increased compared with the control.

Effect of haem oxygenases on Fas ligand in tightly bound PGCs by flow cytometric analysis

To determine whether Fas ligand was induced by haemin and whether this effect was inhibited by ZnPP IX in tightly bound PGCs, we examined the expression of Fas ligand protein by flow cytometry. Representative histograms of the number of tightly bound PGCs staining with anti-Fas-ligand antibody are shown in Figure 9. PGCs stained with anti-Fas-ligand antibody had greater fluorescence intensity than negative controls (grey area), indi-

cating that Fas ligand protein was expressed in tightly bound PGCs at 24 h of culture. PGCs staining with anti-Fas-ligand antibody demonstrated greater fluorescence intensity when treated with haemin, compared with untreated controls (Fig. 9A). The inhibitory effect of ZnPP IX on the haemin-induced increase in Fas ligand protein in tightly bound PGCs was also investigated. Fluorescence intensity in PGCs staining with anti-Fas-ligand antibody was decreased by treatment with ZnPP IX in the presence of haemin, indicating that the increase in Fas ligand produced by haemin was inhibited by ZnPP IX (Fig. 9B).

We calculated the relative amount of fluorescence intensity in each treatment group compared with the control. The results from three separate experiments are

Figure 7 Effect of haem oxygenases on apoptosis in tightly bound porcine granulosa cells (PGCs). (A) Representative DNA histograms in tightly bound PGCs treated with haemin (haem oxygenase substrate) or both haemin and zinc protoporphyrin IX (ZnPP IX; haem oxygenase inhibitor), at 24 h of culture. (B) Mean percentage of apoptotic cells with different concentration of haemin or ZnPP IX, or both. The proportion of apoptotic cells was significantly increased by haemin 1 μM compared with control in tightly bound PGCs, and haemin-induced apoptosis was completely inhibited by ZnPP IX 1 μM. Data are means ± s.d. of four independent experiments. $P < 0.05$ compared with: *control, #treatment with haemin 1 μM.

summarized in Figure 9C. The mean fluorescence intensity was increased by haemin compared with control in tightly bound PGCs, and ZnPP IX inhibited the haemin-

induced increase in a dose-dependent manner. These results suggest that haem oxygenase might regulate the expression of Fas ligand protein in tightly bound PGCs.

Figure 8 Effect of haemin on Fas ligand expression in porcine granulosa cells (PGCs). (A) Western blotting analysis of Fas ligand using polyclonal antibody against proteins extracted from PGCs treated with haemin. Anti-Fas-ligand antibody reacted with a protein band of 38 kDa (approximate molecular mass of porcine Fas ligand). (B) Densitometric analysis of Fas ligand protein in PGCs treated with haemin. Bars represent arbitrary units and are shown as the means $\pm$ S.D. of three independent experiments. * $P < 0.05$ compared with control.

Discussion

This study demonstrated that two haem oxygenase isozymes, inducible HO-1 and constitutive HO-2, are expressed in granulosa cells in the porcine ovary; HO-1 protein was mainly present in granulosa cells in the atretic follicle, whereas HO-2 protein was constitutively observed in granulosa cells in the healthy follicle. Furthermore, this study demonstrated, for the first time, the detection of both haem oxygenase isozymes in freshly isolated PGCs during follicular development by RT-PCR and western blotting, and the presence of both isozymes in the two subpopulations of PGCs (tightly bound and weakly associated GCs) that were freshly isolated (Fig. 4) and cultured (data not shown), by flow cytometric analysis.

Porcine granulosa cells consist of two subpopulations, tightly bound and weakly associated (Howard & Ford 1994). Tightly bound PGCs originate from the mural layers that border the basement membrane of the follicles, whereas weakly associated PGCs originate from the antral layers (Howard & Ford 1994). We were able to characterize two subpopulations of PGCs by flow cytometry and dot plot analysis, comparing forward scatter and side scatter, in both freshly isolated and cultured PGCs. We found that there was a different apoptotic response to serum starvation in tightly bound and weakly associated PGCs. After 48 h of preincubation in medium containing 10% FCS, the proportion of apoptotic cells was less than 1% in tightly bound PGCs and approximately 6% in weakly associated PGCs. The proportion of apoptotic cells in tightly bound PGCs increased to approximately 10% at 24 h under serum starvation. However, in weakly associated PGCs, the proportion of apoptotic cells dramatically

increased to approximately 90% at 24 h under the same conditions. The two subpopulations of PGCs have different characteristics regarding steroid production, mitotic potential and protein content (Marrone & Crissman 1988, Howard & Ford 1994, Duda *et al.* 1999); this is the first report of different apoptotic responses to serum starvation between the two subpopulations of PGCs.

We determined that the rate of apoptosis was significantly increased in tightly bound PGCs by treatment with haemin, and that ZnPP IX, a haem oxygenase inhibitor, inhibited haemin-induced apoptosis. However, the proportion of apoptotic cells was not altered by treatment with haemin in weakly associated PGCs. Because haemin and ZnPP IX were used previously as, respectively, a haem oxygenase substrate and a haem oxygenase inhibitor, to investigate the effect of haem oxygenase on various phenomena in many organs (Eyssen-Hernandez *et al.* 1996, Ye & Laychock 1998, Duckers *et al.* 2001, Alexandrescu & Lawson 2003), we used both compounds in this study. Although high concentrations of ZnPP IX decrease both haem oxygenase and NOS activities, it had been reported previously that, in concentrations lower than 1 μ M, it was able to inhibit haem oxygenase activity but did not affect NOS activity (Appleton *et al.* 1999). At the range of concentration used in this study, ZnPP IX was thus considered to be specific for haem oxygenase. On the basis of these findings, we speculate that products catalysed by haem oxygenase may be apoptotic factors in tightly bound PGCs.

Haem oxygenases are present in a number of cells and tissues, and CO, iron and bilirubin, which are formed from the haem ring by these enzymes, are recognized as apoptotic or antiapoptotic molecules in several systems.

Figure 9 (A) Representative histograms of cells staining with anti-Fas-ligand antibody in tightly bound porcine granulosa cells (PGCs) treated or not treated with haemin. PGCs staining with anti-Fas-ligand antibody had greater fluorescence intensity after treatment with haemin compared with control. (B) Representative histograms of cells staining with anti-Fas-ligand antibody in tightly bound PGCs treated or not treated with ZnPP IX in the presence of haemin. PGCs staining with anti-Fas-ligand antibody had less fluorescence intensity after treatment with ZnPP IX compared with cultures treated with 1 μ M haemin. (C) Mean fluorescence intensity in PGCs treated with haemin or both haemin and ZnPP IX (relative to control). The fluorescence intensity was increased by haemin compared with control in tightly bound PGCs, and ZnPP IX inhibited the increase induced by haemin in a dose-dependent manner. Bars represent the means $\pm$ S.D. of three independent experiments. $P < 0.05$ compared with: *control, #treatment with haemin 1 μ M.

In a murine endothelial cell line, CO generated by HO-1 acts as an antiapoptotic molecule that can suppress cell apoptosis by activation of p38 MAPK (Brouard *et al.* 2000). In murine fibroblasts, HO-1 shows a protective role against cell apoptosis induced by serum deprivation, by reducing intracellular iron concentrations (Ferris *et al.* 1999). Furthermore, in rat cultured pancreatic islets, haem oxygenase provides a protective role against the actions of IL- β -induced NO, which induces cell apoptosis and inhibits insulin release (Ye & Laychock 1998). Conversely, adenovirus-mediated transfer of HO-1 stimulates apoptosis in rat vascular smooth muscle cells through bilirubin, a product of the catalysis of haem by haem oxygenase (Liu

et al. 2000). In the male reproductive organ, HO-1 protein was present in a low concentration in normal testes, but cadmium chloride increased HO-1 activity in Leydig cells, to cause germ cell apoptosis by CO (Ozawa *et al.* 2002). Thus bilirubin is an apoptotic factor in vascular smooth muscle cells and CO induces apoptosis in germ cells in testis, respectively. Although we showed that haem oxygenase products augmented granulosa cell apoptosis, further experiments are needed to determine which factor produced by haem oxygenase stimulates this apoptosis in porcine ovary.

We demonstrated that Fas ligand was significantly increased by haemin and decreased by ZnPP IX in PGCs,

which suggests that haem oxygenase has the ability to upregulate Fas ligand protein in tightly bound PGCs. Follicular atresia and luteolysis are generally considered to occur by apoptosis. Recent reports characterizing the molecular mechanisms underlying this process demonstrate the role of several factors, including intracellular calcium concentration, MAPK, adenosine triphosphatase and extracellular-signal regulated kinase (Gebauer *et al.* 1999, Peluso *et al.* 2001, 2003, Park *et al.* 2003). The Fas/Fas ligand system has been identified as a mediator of granulosa cell apoptosis in the rat ovary (Hakuno *et al.* 1996, Kim *et al.* 1999), and treatment with monoclonal anti-human-Fas antibody induces apoptosis in cultured human granulosa/luteal cells (Quirk *et al.* 1995). Fas-mediated cell-death pathways are considered to be central to the induction of follicular atresia (Quirk *et al.* 1995, 2000, Hakuno *et al.* 1996, Kim *et al.* 1999). Our data demonstrate that haem oxygenase has the ability to increase the production of Fas ligand protein, after granulosa cell apoptosis, in tightly bound PGCs, which suggests that products generated by haem oxygenase may be autocrine/paracrine factors that promote granulosa cell apoptosis.

Haem oxygenases are induced by various factors (Otterbein & Choi 2000). Several authors have shown that HO-1 was induced by heat shock, heavy metals, hypoxia and endotoxin (Eyssen-Hernandez *et al.* 1996, Carraway *et al.* 1998, Panchenko *et al.* 2000, Stuhlmeier 2000). Furthermore, HO-1 was induced by TNF- α (Terry *et al.* 1998), which is known to stimulate granulosa cell apoptosis (Kaipai *et al.* 1996). In porcine granulosa cells, we have demonstrated that HO-1 was mainly expressed in granulosa cells in the atretic follicle, and was present in freshly isolated and cultured granulosa cells, but we have not studied the nature of the factors that induced HO-1. HO-1 may be induced by stress factors, and may play a part in controlling the female gamete in stressful environments through granulosa cell apoptosis in the ovaries, as occurs in testes (Ozawa *et al.* 2002). Further investigation of the regulatory mechanisms of the expression of haem oxygenase and the possible role of haem oxygenase in atresia is warranted.

In conclusion, the present study demonstrates that two haem oxygenase isozymes, inducible HO-1 and constitutive HO-2, can be identified in both subpopulations of granulosa cells – tightly bound and weakly associated – throughout follicular development, and that HO-1 was strongly expressed in granulosa cells in the atretic follicles. A product of the catalysis of haem by haem oxygenase augments apoptosis in tightly bound PGCs (which originate from the mural layers that border the basement membrane of the follicles), possibly by upregulation of the Fas ligand protein. Although further studies are needed to prove the direct relationship between haem oxygenases and follicular atresia, the products produced by haem oxygenase, namely CO, bilirubin and ferritin, can be

considered to be autocrine/paracrine factors of apoptosis in PGCs, and may be important factors in atresia.

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