Haem oxygenase augments porcine granulosa cell apoptosis in vitro

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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 stimuli 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 cerebral 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 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 intracellular 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-1β that induce 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 21α-hydroxylase and mitochondrial 11β-hydroxylase (Maines & Trakshel 1992). In rat testes, SnPP IX also inhibits the activities of cytochrome P450 and microsomal 17α-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 (Alexandreanu & 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 populations of PGCs, weakly associated and tightly bound, which are distinguished by size and have different functional characteristics (Marrone & Crisman 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 µm 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 µg/ml deoxyribonuclease (DNase) (Sigma)/D-PBS containing 0·133 mg/ml calcium chloride 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 RNA was determined by spectrophotometry at 260 and manufacturer’s instructions. The quantity of extracted total RNA was isolated from PGCs using RNeasy mini kit and one-tenth DNase-treated RNA using the SuperScript First-Strand Synthesis System (Life Technologies Inc.), and then was 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.

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 (Affinity Research Product Ltd, Exeter, Devon, UK) or zinc protoporphyrin IX (ZnPPIX), 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% Nonidet P-40, 0·1% deoxycholic acid, 10 µM phenyl methylsulphonyl fluoride, 0·5 mM apoprotein, 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 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 15% 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
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.
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 µM to 1 µM, but was not altered by culture with less than 0.01 µM of this agent (data not shown). Culture with haemin at 0.1 µM and 1 µM 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 µM 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 µM 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
treatment, the mean percentage of apoptotic cells was low (0.27 ± 0.17%, mean ± 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 μM 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 ± 3.4% compared with 10.0 ± 2.7%, P < 0.05).

Representative DNA histograms of weakly associated PGCs treated with or without 1 μM 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 μM 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 ± 3.1%), and was significantly increased at 4 h of culture in serum-free conditions (50.0 ± 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 μM 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.
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 ± 1.9% compared with 9.8 ± 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
quantity of Fas ligand protein from PGCs treated with 1 µM 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), indicating 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...
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 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; haemoxygenase inhibitor), at 24 h of culture. (B) Mean percentage of apoptotic cells treated 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.
Discussion

This study demonstrated that two haem oxygenase iso-
zymes, 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 associ-
ated 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 charac-
terize 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 associ-
ated PGCs, the proportion of apoptotic cells dramatically 
increased to approximately 90% at 24 h under the same 
conditions. The two subpopulations of PGCs have differ-
cent 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 signifi-
cantly increased in tightly bound PGCs by treatment with 
haemin, and that ZnPP IX, a haem oxygenase inhibitor, 
inhibited haemin-induced apoptosis. However, the pro-
portion 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 
oxidase 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, 
Alexandreanu & 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 µM, 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.
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 augumented 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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