AG 205, a PGRMC1 Antagonist, Ablates Progesterone's Ability to Block Oxidative Stress-Induced Apoptosis of Human Granulosa/Luteal Cells

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Summary sentence: The PGRMC1 antagonist, AG 205, blocks the ability of P4 to inhibit H_2O_2-induced apoptosis by disrupting PGRMC1 dimers/oligomers and inducing the expression of the apoptosis inducing BH3-only protein, Harakiri.

Key Words: Apoptosis, Granulosa cells, PGRMC1, PGRMC2, Progesterone

Abstract

The present studies were designed to determine whether progesterone (P4)-Progesterone Receptor Membrane Component 1 (PGRMC1) signaling is able to attenuate the apoptotic effects of oxidative stress induced by hydrogen peroxide (H_2O_2). To achieve this goal, freshly isolated human granulosa/luteal cells were maintained in culture. After several passages, the cells were treated with H_2O_2, which induced apoptosis within 2.5 h, while simultaneous treatment with P4 attenuated the apoptotic action of H_2O_2. AG 205, a PGRMC1 antagonist, eliminated P4's ability to prevent H_2O_2-induced apoptosis. AG 205 neither affected PGRMC1's cytoplasmic localization nor its interaction with PGRMC2, but appeared to reduce its presence within the nucleus. AG 205 also 1) increased the monomeric and decreased the higher molecular weight forms of PGRMC1 (i.e. dimers/oligomers) and 2) altered the expression of several genes involved in apoptosis. The most dramatic change was an approximate 8-fold increase in Harakiri (*Hrk*) mRNA. However, AG 205 did not induce apoptosis in the absence of H_2O_2. Taken together these observations suggest that the higher molecular weight forms of PGRMC1 likely account in part for PGRMC1's ability to suppress the expression of *Hrk*. HRK is a BH-3 only member of the BCL2 family that promotes apoptosis by binding to and antagonizing the anti-apoptotic action of BCL2 and BCL2-like proteins. It is likely then that PGRMC1's ability to suppress *Hrk* is part of the mechanism through which P4-PGRMC1 signaling preserves the viability of human granulosa/luteal cells.

Introduction

Progesterone (P4) plays an essential role in regulating female reproductive function and fertility, influencing components of the entire reproductive axis [1, 2]. In the ovary, the best-defined role for P4 is its requirement for ovulation [3]. In primates, P4's requirement for ovulation is revealed through the use of drugs, which inhibit its synthesis [4]. In mice the genetic ablation of the classic nuclear receptor (PGR) conclusively demonstrates that P4 mediates its effect on ovulation through this receptor because these mutant mice do not ovulate [3]. In addition to ovulation, P4 plays an essential role in the formation and maintenance of the corpus luteum. This is an extremely important aspect of P4's intraovarian function, since the premature demise of the corpus luteum leads to the reduction in serum P4 levels and ultimately the failure of implantation and/or premature termination of pregnancy [5, 6]. P4's anti-apoptotic action is also observed in the granulosa cells of various sized follicles [7] and luteinizing granulosa cells of the preovulatory follicle [4]. Interestingly, human chorionic gonadotropin (hCG) induces luteinizing granulosa cells within the preovulatory follicle of non-human primates to undergo apoptosis if P4 synthesis is inhibited using trilostane [4]. The apoptotic action of hCG/trilostane is overcome by supplemental progestin. Thus, P4's anti-apoptotic effect on luteinizing granulosa cells is essential for the formation of the corpus luteum [4].

As luteinization progresses, serum gonadotropins induce the expression of steroidogenic genes [8] thereby increasing P4 levels, which function to maintain the viability of luteal cells [9]. This positive feedback mechanism is in effect until the late luteal phase, when the capacity of the luteal cells to respond to gonadotropins is reduced, P4 synthesis decreases and the luteal cells undergo apoptosis [4]. The mechanism responsible for the decreased sensitivity to gonadotropins has not been clearly defined and may be due to a putative increase in ovarian prostaglandin F2α [10]. Importantly, the reduced ability to respond to gonadotropins is not the only factor that leads to luteal cell death, because luteal cells are also exposed to apoptotic

factors during the mid to late luteal phase of the menstrual cycle. One such factor is reactive oxygen species (ROS) that are generated by leukocytes that invade the corpus luteum [10, 11].

With the decline in P4 during the menstrual cycle and the increasing apoptotic effects of the ROS, the luteal cells undergo apoptosis and the corpus luteum gradually regresses. If conception occurs, then the corpus luteum is maintained even in the presence of ROS death-inducing activity. This is achieved by hCG, which is derived from the trophoblasts of the implanting embryo and acts to increase StAR expression and ultimately P4 synthesis [12]. This increase in P4 could potentially block the ROS-activated apoptotic pathway. Thus, determining whether P4 can attenuate these apoptotic pathways is absolutely critical for defining the mechanism involved in the maintenance of the corpus luteum. To this end, we isolated granulosa/luteal cells from women undergoing in vitro fertilization. These cells were maintained in culture for several passages and then used in a series of experiments to determine whether P4 can inhibit ROS (e.g. hydrogen peroxide; H_2O_2)-induced human granulosa/luteal cell apoptosis. In addition the present studies extend our previous work in which PGRMC1 siRNA attenuated P4's anti-apoptotic actions in human granulosa/luteal cells [9] by using the PGRMC1 antagonist, AG 205. This approach was used because PGRMC1 exists as different molecular weights, with siRNA depleting all the molecular weight forms. Since the different molecular weight forms of PGRMC1 could have different functions, we opted to assess the effect of AG 205, which allows PGRMC1 to remain within the cell.

Materials and Methods

Human Granulosa/Luteal Cell Isolation, Culture, and Propagation

The process for granulosa/luteal cell isolation and culture has been detailed in our previous studies [13]. Briefly, follicular aspirates were collected from patients undergoing in vitro fertilization at the Center for Advanced Reproductive Services at the University of Connecticut Health Center. Samples were deidentified, thereby precluding any link to clinical information. As

such the Institutional Review Board of the University of Connecticut Health Center declared that this protocol was exempt as it was considered non-human research.

Once the oocytes were removed, the follicular aspirates were transported to the research laboratory where the follicular fluid was centrifuged and the cell pellet aspirated, resuspended in phosphate-buffered saline (PBS), and layered with Histopaque-1077 gradient (catalog number 10771; Sigma-Aldrich). Following centrifugation at 400 x g for 30 min [9, 14], the cohesive band of cells within the Histopaque was pipetted onto an inverted 40 µm cell strainer (catalog number 08-771-1; Fisher Scientific) overlying a 50 ml tube [15]. The aspirate was washed once with PBS to remove macrophages, lymphocytes, and monocytes. The strainer was then properly positioned into a new 50 ml tube and washed with PBS. The fluid was centrifuged at 250 x g for 10 min and the pellet resuspended in PBS. After an additional 2 centrifugations, the isolated granulosa/luteal cell pellet was resuspended in 0.5% trypsin and incubated at 37° C for 10 minutes, followed by the addition of fetal bovine serum (FBS)-supplemented medium to stop the reaction, and a final centrifugation at 250 x g x 10 min. The pellet was resuspended in Dulbecco modified Eagle medium (DMEM) supplemented with 1% ITS (insulin-transferrin-selenium; catalog number I3146; Sigma-Aldrich), 0.01% gentamicin (catalog number G1397; Sigma-Aldrich), and 10% FBS. Cells from individual patients were cultured on Matrigel (catalog number 354230; BD Bioscience)-coated T-25 culture flasks until they were confluent. These cells were then harvested, pooled, and maintained in culture. After 5-8 passages, the cells were frozen and stored in liquid nitrogen for use in all subsequent experiments. As needed, human granulosa/luteal cells were thawed and cultured in Matrigel-coated T-25 or T-75 culture flasks. When confluent, the cells were harvested and plated on non-matrigel coated culture dishes for 24-48 h prior to any treatment. Cells were discarded after 13 passages.

Microplate Reader Based Assay for Cell Viability

Granulosa/luteal cells were harvested and counted using a hemacytometer. These cells were subsequently plated in a 96-well black, clear-bottomed plate at a density of 5×10^4 in 100ul of steroid-free serum (catalog number 30068-03; Thermo Scientific)- supplemented medium and cultured overnight. In our initial attempt to identify cells undergoing apoptosis [16, 17], cells were fixed and stained with Annexin V-CY3 per the manufacturer's recommendations (catalog number K102-35; Biovision). Apoptotic cells were identified by fluorescent microscopy, but the emission signal was not detectable using a microplate reader (Biotek Synergy 2 Multimode Reader; red fluorescence, 540 nm excitation, 508 nm emission). In order to develop a microplate-based apoptosis assay, cells were costained with Annexin V-CY3 and 488 dead cell stain [18], in accordance with the manufacturer's protocol (catalog number L34969; Life Technologies). Costained cells were then sequentially observed under epifluorescent microscope using the RFP and FITC filter set. This study revealed that those cells stained with Annexin V-CY3 also stained with 488 dead cell stain. Moreover, the 488 signal (green fluorescence, 490 nm excitation, 525 nm emission) was sufficiently bright enough to be detected with the microplate reader. Using this microplate assay, pilot studies revealed that the 150 μM H_2O_2 for 2.5 h at 37° C was optimal for inducing human granulosa/luteal cell apoptosis.

Based on this, apoptosis was assessed using the following microplate assay. Granulosa/luteal cells were harvested and plated as previously described. After overnight incubation, media was removed and cells were washed once with Hank's balanced salt solution (HBSS). Cells were then cultured at 37°C for 150 min in HBSS with 1 μM P4, 150 μM H_2O_2, or both P4 and H_2O_2. Controls were treated with HBSS and an equivalent volume of vehicle (100% ethanol). The dose of 1 μM P4 was selected because P4 concentrations in follicular fluid of non-stimulated follicles are ≥ 1 μM [19] and P4 levels after hCG stimulation are approximately 10 μM [20]. In one study, P4 was added 15 min after H_2O_2. After a 2.5 h incubation period, the 96-well plate was centrifuged at 250 x g for 10 min and the media was discarded. Cells were

then stained with 488 dead cell stain in HBSS according to the manufacturer's protocol and placed on a shaker at room temperature for 30 min, protected from light. The plate was again centrifuged and the cells were washed with HBSS. Cells were fixed with 4% formaldehyde in HBSS for 15 min at room temperature, protected from light. Following an HBSS wash, nuclei were stained with DAPI to determine the total number of cells (4', 6-diamidino-2-phenyl-indole, dihydrochloride; catalog number D9564; Sigma-Aldrich, 0.2 µg/ml HBBS for 5 minutes; blue fluorescence 350 nm excitation, 470 nm emission). After a final HBSS wash, 50µl of HBSS was added to each well and the plate was read with the microplate assay as detailed above. Dead cell/ total cell ratio was obtained to estimate the effect of various treatments on the rate of apoptosis.

Effect of the PGRMC1 Antagonist (AG 205) on P4's Ability to Inhibit H_2O_2-induced Apoptosis

These studies were conducted as outlined in the above paragraph. However, following the initial 24 h incubation period, the media was replaced with fresh steroid-free serum-supplemented medium in the presence of either 50 µM AG 205 (catalog number A1487; Sigma-Aldrich) or an equivalent volume of DMSO for the controls. The 50 µM dose of AG 205 was selected based on the IC_{50} established by Ahmed et al [21]. The cells were then incubated overnight and the remainder of the experiment proceeded as above.

Effect of AG 205 on the Localization of PGRMC1 and PGRMC2

Cells were cultured at a density of 4 x 10^5 on cover glass overnight in steroid-free serum-supplemented medium. The media was subsequently replaced with fresh steroid-free serum-supplemented medium in the presence of either 50 µM AG 205 or an equivalent volume of DMSO for the controls. Following an additional overnight incubation at 37° C, the cover glasses were washed once with HBSS and fixed with 4% paraformaldehyde for 7 min on ice. Cells were washed with PBS for three times for 5 min each, followed by permeabilization with 0.1% Triton X-100 in PBS for 7 min. Following another set of PBS washes, the cells were incubated in 3%

bovine serum albumin (BSA) for 30 min at 4° C for blocking. The primary antibodies (PGRMC1: catalog number 98680, Santa Cruz; PGRMC2: catalog number ab113646, abcam) were added at a concentration of 1:100 in 0.1% BSA and incubated at 4° C overnight. Negative controls were incubated with 0.1% BSA in place of the primary antibody. The following day, the appropriate species-specific secondary antibodies were added to all cells (1:800 in 0.1% BSA) following three PBS washes of 5 min each. Cells were incubated for 1 h at room temperature on the shaker, protected from light. Following a final set of PBS washes, each cover glass was mounted on a slide with ProLong Anti-fade mounting media (Invitrogen-Molecular Probes) and subsequently imaged with a Zeiss Axio Observer inverted microscope (Carl Zeiss MicroImaging Inc.) with a Lumen 200 Fluorescence Illumination System (Prior Scientific Inc.) and a QImaging Retiga EXi CCD digital camera (QImaging).

Effect of AG 205 on the Interaction between PGRMC1 and PGRMC2

Cells were initially prepared and incubated with the primary antibodies as described for immunofluorescence. The in situ Proximity Ligation Assay (PLA) was conducted in accordance with the manufacturer's protocol (Doulink II Fluorescence; Sigma-Aldrich). In the PLAs, the secondary antibodies were labeled with complimentary DNA probes. An interaction between PGRMC1 and PGRMC2 was detected when the two complimentary probes were close enough in proximity to hybridize and form a double-stranded DNA. The double-stranded DNA was subsequently amplified and detected by a fluorescent probe that appeared as an isolated red dot.

The degree of interaction between PGRMC1 and PGRMC2 was assessed by determining the number of red dots per cell. This was done by identifying individual dots using the threshold function and quantifying the number of cells with the iVision Image Acquisition and Analysis Software. Negative controls were also analyzed in this manner to estimate the background, which was subsequently subtracted from the total number of red dots observed in treatment groups. The resulting value was divided by the number of cells observed in each field.

For each replicate, three to four randomly selected fields from each treatment group were analyzed and the average number of dots/cell determined. Because the average number of dots/cell varied between the two experiments, values were expressed as a fold-increase over the DMSO control from each replicate. The fold increase data was used to generate a mean ± one standard error for each treatment group.

Western Blot Analysis of PGRMC1

Cells were cultured in non-Matrigel coated T25 flaks until confluent. Media was replaced with fresh steroid-free serum-supplemented medium in the presence of either 50 µM AG205 or an equivalent volume of DMSO for the controls. Following overnight incubation at 37° C, cells were washed once with HBSS and lysed in RIPA buffer (50mM Tris, 150mM sodium chloride, 1 mM EDTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate, pH 7.0), which was supplemented with complete protease inhibitor cocktail (catalog number 11697498001; Roche) and phosphatase inhibitor cocktail (catalog number 524624, Calbiochem). Lysates were placed on ice x 30 min on the shaker, followed by centrifugation at 250 x g for 10 min. Protein concentration was determined using the Pierce BCA protein assay kit (catalog number 23225; Thermo Scientific). Protein levels were determined using previously published protocols [22] using antibodies to either PGRMC1 (1:500 dilution; catalog number HPA 002877, Sigma Aldrich) or GAPDH (1:500 dilution; catalog number MAB374, Millipore). All blot protocols included a negative control in which the primary antibody was omitted.

To assess PGRMC1 expression in the presence of a reactive oxygen species, cells were cultured as above. The treatment group was incubated in the presence of 150 uM H_2O_2 for 150 min. Controls were incubated with HBSS and an equivalent volume of 100% ethanol. The remainder of the Western blot proceeded as described above.

iVision Image Acquisition and Analysis Software (BioVision Technologies) was used to monitor changes in the expression of the different molecular weight forms of PGRMC1. Briefly,

the area of each individual band was identified as a region of interest and then its average band density determined. Similarly, an adjacent area was used to calculate average background density. The background density was then subtracted from the average density of each band. For each sized band, the value of the control was use to determine the fold increase induced by H_2O_2. Values from each of 4 experiments were averaged to generate a means ± one standard error.

Effect of AG 205 on the Expression of Genes that Regulate Apoptosis

Cells were cultured and treated with AG 205 or an equivalent amount of DMSO for the controls as described above. Following overnight incubation at 37° C, an RNeasy Plus Mini-Kit (catalog number 74134; Qiagen) was used to isolate total RNA per manufacturer's instructions. The amount of RNA isolated was determined using a Nanodrop spectrophotometer and, for each sample, an equal amount of RNA was converted to cDNA using the RT^2 First Strand Kit (catalog number 330401; Qiagen). Using the cDNA as a template, real-time PCR was performed using the RT^2 Profiler PCR Array for human apoptosis (catalog number 330231; Qiagen) and corresponding RT^2 SYBR® Green qPCR Mastermix (catalog number 330502; Qiagen). Forty cycles were run in a Bio-Rad CFX96 real-time cycler with the following conditions: enzyme activation for 10 min at 95° C, denaturation for 15 sec at 95° C, and annealing/extension at 60° C for 1 min. Gene expression was evaluated with the Bio-Rad CFX96 software using the provided $2^{-\Delta\Delta CT}$ method. mRNA levels of the 84 genes of interest were compared to 5 housekeeping genes.

Effect of AG 205 on the Localization of HRK and Cytochrome c

Cells were cultured on 35 mm dishes and treated with AG 205 or DMSO overnight as previously outlined. Immunofluorescence then proceeded as detailed above using the primary antibody for HRK at a dilution of 1:100 (catalog number 59907; abcam). In order to assess the mitochondrial localization of cytochrome c, cells were incubated for 30 min at 37°C in the

presence of 200 nM of Mitotracker Orange CMTMRos (catalog number M7510; Invitrogen), per the manufacturer's protocol. Cells were then stained for cytochrome c using a primary antibody provided by Cell Signaling Technologies (catalog number 12963) at a dilution of 1:300. Negative controls were incubated with 0.1% BSA in place of the primary antibody.

In order to monitor the effect of AG 205 on the total amount of cytochrome c, lysates were prepared after treatment with either DMSO or AG 205 as previously described. Fifty µg aliquots of lysate were then loaded onto a 4 to 20% gradient gel (catalog number 456-1094; Bio-Rad). Western blots were probed using rabbit anti-cyctochrome c antibody (1:1000 dilution; catalog number 11940; Cell Signaling Technologies) and processed as previously described.

Statistical Analysis

For all experiments, treatments were conducted in duplicate or triplicate and were replicated a minimum of two times, except for the apoptosis assays in which each treatment was replicated 4 to 8 times per experiment. Values from each experiment were pooled to generate a mean ± standard error. A Student t-test was used to assess the differences between two treatment groups. When comparing more than two groups, a two-way analysis of variance, followed by a Fisher's least significant difference post hoc test was used. $P \leq 0.05$ was considered to be significantly different regardless of the statistical test. All statistical analyses were completed using PRISM software (Version 6.0; GraphPad).

Results

Pilot studies revealed that the optimal concentration of H_2O_2 that induced apoptosis after 2.5 h was 150 µM, consistent with the findings of Vega et al [11]. This was demonstrated by Annexin V- CY3 staining, which detected cells in the early stages of apoptosis [16, 17]. However, the emission signal associated with Annexin V-CY3 staining was not detectable using a microplate reader. In order to develop a microplate apoptosis assay, cells were subsequently costained with Annexin V-CY3 and 488 dead cell stain [18], followed by counter staining with

DAPI. A comparison of **Figure 1A** and **B** revealed that the cells that stained for Annexin V-CY3 (red fluorescence in **Figure 1A**) also stained with 488 dead cell stain (green fluorescence in **Figure 1B**). This green fluorescent signal was strong enough to be read in a microplate reader. DAPI was used to stain the nuclei of all cells and, when assayed in a microplate reader, the intensity of the DAPI staining was directly proportional to the number of cells (r=0.99, data not shown). Thus a ratio of 488 dead cell stain (green fluorescence) to the DAPI staining (blue fluorescence) allowed for the estimation of the dead to total cell ratio. As illustrated in **Figure 1C**, H_2O_2 induced a 7.8 ± 0.7 fold increase in the dead cell to total cell ratio. Moreover, the addition of P4 attenuated the apoptotic action of H_2O_2 **(Figure 1D)**, while P4's anti-apoptotic action was eliminated by pretreatment with AG 205 for 24 h **(Figure 1D)**.

Because siRNA-based studies demonstrate that P4's actions are dependent on PGRMC1 [9], the effect of AG 205 on the cellular localization of PGRMC1 was assessed. In control cells, PGRMC1 was localized to the cytoplasm as well as the nucleus of many cells. In contrast after treatment with AG 205, PGRMC1 was prominently located in the cytoplasm, with some nuclei apparently devoid of PGRMC1 staining (67 ± 8% with nuclear PGRMC1 staining in the DMSO treatment group vs 31 ± 10% for AG 205 treatment group; n=3; p < 0.05; compare **Figures 2A,B** with **2C,D**). Staining as indicated by green fluorescence was not detected in the absence of the PGRMC1 antibody **(Figures 2E, F)**. Since P4's anti-apoptotic actions are also dependent on PGRMC2 [13, 23, 24], the effect of AG 205 on the cellular localization of PGRMC2 was assessed. PGRMC2 was found almost exclusively in the cytoplasm **(Figure 3A)**. This localization was unchanged after 24 h treatment with AG 205 **(Figure 3B)** and not detected when staining was done in the absence of the PGRMC2 antibody **(Figure 3C)**.

In rat spontaneously immortalized granulosa cells endogenous PGRMC1 and PGRMC2 interact in order for P4 to affect its anti-apoptotic action [24]. Because of this, the effect of AG 205 on the interaction between PGRMC1 and PGRMC2 was assessed. Under control conditions the interaction between PGRMC1 and PGRMC2 was readily detectable **(Figure 4A)**.

Moreover, their interaction was significantly enhanced following treatment with AG 205 (Compare **Figure 4A** with **4B**) as indicated by AG 205 inducing a 2.3± 0.47 fold increase in the number of dots/cell (p < 0.05). Note that red dots were rarely observed in the negative control **(Figure 4C)**.

PGRMC1 has been shown to exist as a low molecular weight (monomer) form of about 27 kDa and higher molecular weight forms ≥ 50 kDa [25, 26]. Given that higher molecular weight forms of PGRMC1 tend to be located in the nucleus [26], the effect of AG 205 on presence of the higher molecular weight forms of PGRMC1 relationship was assessed by Western blot. In the presence of AG 205, the abundance of the lower molecular weight form of PGRMC1 increased and the higher molecular weight forms decreased **(Figure 5A)** with GAPDH levels being similar regardless of treatment **(Figure 5B)**. In addition H_2O_2 increased all the molecular weight forms of PGRMC1 as compared to controls **(Figure 6A)** with **Figure 6B** confirming that equal amounts of protein were loaded onto the gel. The H_2O_2-induced increase in PGRMC levels was statistically significant for those bands corresponding to ≈50 kDa and ≈75 kDa **(Figure 6C)**. Although H_2O_2 induced the higher molecular weight forms for PGRMC1 within 2.5 h, P4 treatment did not inhibit H_2O_2-induced apoptosis if given 15 min after H_2O_2 (**Figure 7**).

Despite interfering with the nuclear localization of PGRMC1, pretreatment with AG 205 did not adversely affect the morphology of the cells **(Figure 8A compared to Figure 8B)**. Similarly, exposure to AG 205 did not influence the expression of most of the apoptosis-related genes monitored in this study **(Figure 8C)**. There were, however, several genes whose expression increased by 1.5 to 3 fold compared to controls (p< 0.05) but the most striking change associated with AG 205 treatment was a 6 to 8 fold increase in *Hrk* mRNA (p< 0.05) **(Figure 8D)**. In addition the increase in *Hrk* mRNA was confirmed by detecting increased levels of HRK within the cytoplasm of AG 205 treated cells **(Compare Figure 8E with 8F)**. Negative controls did not detect any green fluorescence **(Supplemental Figure 1)**.

Cytochrome c was detected in the cytoplasm of DMSO-treated cells, mainly as punctate

foci **(Figure 9B),** whose localization corresponded to areas enriched in mitochondria as revealed by MitoTracker staining **(Figure 9A).** After AG 205 treatment some cytochrome c was still detected as punctate foci **(Figure 9E),** indicating that it remained co-localized to areas with high concentrations of mitochondria **(Figure 9D)**. However, much of the cytochrome c was present in areas of the cytoplasm devoid of mitochondria, as revealed by a diffuse staining pattern **(Figure 9E)** and the large amount of green fluorescence in merged image of **Figure 9F** compared to **Figure 9C**. Green fluorescence in the negative controls was essentially non-detectable (Data not shown). Although AG 205 treatment altered the distribution of cytochrome c, it did not reduce the overall levels of cytochrome c, as assessed by Western blot **(Figure 10)**.

Discussion

The lifespan of human granulosa and luteal cells is dependent on their exposure to factors that either promote cell viability or induce cell death. One mechanism by which these cells die is through the intrinsic (mitochondrial) apoptotic pathway, which is often induced by exposure to ROS [27]. Conversely, the viability of granulosa cells of various sized follicles as well as luteal cells is maintained by growth factors and hormones including endogenous P4 [4, 7]. Moreover, P4's anti-apoptotic action in both granulosa and luteal cells is dependent in part on PGRMC1 [9, 28]. In the present study, this relationship is explored by exposing cultured human granulosa/luteal cells to the ROS, H_2O_2, in the presence or absence of P4. Under the conditions employed, H_2O_2 induces a several fold increase in apoptotic cells with the simultaneous exposure to P4 attenuating the apoptotic effect of H_2O_2. In addition, the PGRMC1 antagonist, AG 205, completely eliminates the protective action of P4. The effect of AG 205 is predictable given that PGRMC1 siRNA treatment effectively depletes PGRMC1 and eliminates the capacity of P4 to inhibit rodent [9, 26] and human granulosa/luteal cells [21] from undergoing apoptosis. These siRNA-based studies conclusively demonstrate that the presence of PGRMC1 is essential for P4's anti-apoptotic function. However unlike PGRMC1 siRNA approach,

exposure to AG 205 does not deplete PGRMC1. This raises the question as to how AG 205 blocks PGRMC1's action.

AG 205 interacts with the cytochrome b5/heme binding domain of PGRMC1 [21]. This domain is the site within PGRMC1 that interacts with various proteins [29]. The interaction between PGRMC1 and PGRMC2 is an essential component in the mechanism that regulates the entry of human granulosa/luteal cells into the cell cycle [13] and the survival of spontaneously immortalized granulosa cells [24]. AG 205 could disrupt PGRMC1: PGRMC2 interaction and as a consequence alter the cellular localization of either PGRMC1 or PGRMC2. Based on immunocytochemical analysis, AG 205 alters the cellular distribution of PGRMC1 by apparently reducing its presence within the nucleus. However in the presence of AG 205, PGRMC1 as well as PGRMC2 remain in the cytoplasm. Importantly, the interaction between PGRMC1 and PGRMC2 is not only maintained within the cytoplasm but also significantly enhanced. Since AG 205 increases the amount of PGRMC1 monomer ([21], present study), which mainly localizes to the cytoplasm [26, 30], the AG 205-induced increase in the PGRMC1 monomer could account for the increase in PGRMC1: PGRMC2 interaction simply by enhancing the stoichiometric relationship between PGRMC1 and PGRMC2. These observations make it unlikely that AG 205 attenuates P4's anti-apoptotic action by disrupting the interaction between PGRMC1 and PGRMC2.

PGRMC1 also exists as higher molecular weight forms ≥ 50 kDa [14, 31]. These higher molecular weight forms are dimers/oligomers [26, 32, 33] with some undergoing sumoylation [30], thereby accounting for their higher molecular weights. These higher molecular weight forms predominately localize to the nucleus [30], although some PGRMC1 dimers/oligomers are found in the plasma membrane [21, 34]. The finding that AG 205 reduces the higher molecular weight forms of PGRMC1 is consistent with fewer AG 205-treated cells with PGRMC1 within their nuclei. While PGRMC1: PGRMC2 interaction within the cytoplasm is required to maintain cell viability [13, 24], it appears that cytoplasmic PGRMC1 and its interaction with PGRMC2 is

not sufficient to prevent H_2O_2-induced apoptosis. Therefore the higher molecular weight forms of PGRMC1 must also play an essential role in the mechanism through which P4 transduces its anti-apoptotic action. This conclusion is also supported by a previous study in which all the endogenous forms of PGRMC1 were depleted and replaced with GFP-tagged PGRMC1, which has a limited capacity to form higher molecular weight forms and does not localize to the nucleus [26]. Under these conditions, the elevated cytoplasmic levels of GFP-tagged PGRMC1 are not sufficient to mediate P4's anti-apoptotic effects [26].

Insight into the function of PGRMC1 is suggested by the findings that within 2.5 h of H_2O_2 exposure, PGRMC1 levels of all molecular weight forms are increased with statistically significantly increases in the ≈ 50 and ≈ 75 kDa forms. This may be a common cellular response to stress as exposure to environmental stressors such as dioxin increase PGRMC1 levels [21]. Similarly, granulosa cells isolated from women living in highly polluted environments express higher levels of PGRMC1 [35]. Also ovarian cancer cells up regulate PGRMC1 expression with PGRMC1 predominately localizing to the nucleus [36, 37]. These ovarian cancer cells are more resistant to the toxic effects of chemotherapeutic agents [36, 37]. However in our experimental model, the putative protective action of elevated PGRMC1 levels that are induced in response to stress are ineffective in mitigating H_2O_2's apoptotic action, since P4-PGRMC1signaling must be initiated within 15 min of H_2O_2 treatment.

Recent studies reveal that cytochrome b5/heme binding domain is required for the formation of the high molecular weight forms of PGRMC1 (i.e. dimers/oligomers) [32, 33]. The PGRMC1 dimers/oligomers are the result of a heme molecule binding to the cytochrome b5/heme binding domain of a PGRMC1 monomer with an adjacent heme-PGRMC1 complexes binding to each other [32, 33]. AG 205 competes with heme for the cytochrome b5/heme binding domain of PGRMC1 [21], which would account to the AG 205-induced disruption of the PGRMC1 dimers/oligomers. This is important in that one function of PGRMC1 dimers/oligomers involves directing growth factor receptors such as the epidermal growth factor

(EGF) receptor, to the plasma membrane [32, 34]. EGF is known to activate cell survival pathways [38-40] and is presumably present in the serum-supplemented media used in the present studies. Thus, impeding the plasma membrane localization of the EGF receptor could explain part of AG 205's actions, but this concept remains to be tested experimentally.

The consequences of AG 205 disrupting the PGRMC1 dimers/oligomers also may impact the gene expression profile. Previous studies show that depleting PGRMC1 influences the expression of numerous genes [41]. Interestingly, PGRMC1 appears to primarily function to suppress gene expression as depleting PGRMC1 results in a disproportionate increase in the number of gene transcripts [41]. PGRMC1's tendency to suppress gene expression is confirmed by the present study in that pretreatment with AG 205 increases the expression of 7 of the 84 apoptosis-related genes monitored. Surprisingly, 5 of the 7 genes whose expression is enhanced by AG 205 are known to inhibit apoptosis (i.e. *Bag1, Bcl2a1, Birc3, Mcl1* and *Xiap*) [42, 43]. The observed increase in these genes is statistically significant and might imply that PGRMC1 promotes cell death by suppressing anti-apoptotic genes. However, this would be contrary to PGRMC1's well-documented survival function [29, 44]. It is important to appreciate that expression of these genes is only increased 1.5 to 3 fold. In contrast, AG 205 induces a 7 to 8 fold increase in the mRNA levels of the BH-3 only gene, *Hrk*, which is known to induce apoptosis in several cell types [45-47]. It is likely then that the enhanced expression of *Hrk* overrides any potential effect of the enhanced expression of the 5 survival genes.

Interestingly, in the absence of an apoptotic stimuli (i.e. H_2O_2) AG 205 increases *Hrk* expression but does not immediately lead to cell death, as AG 205-treated human granulosa/luteal cells are morphologically indistinguishable from DMSO-treated control cells. Rather the elevation in *Hrk* expression and its enhanced presence within the cytoplasm likely renders the cells more responsive to the effects of apoptotic agents such as H_2O_2 as well as limiting the ability of P4-PGRMC1 signaling to promote cell survival. This is in contrast to other

cell types in which an apoptotic stimulus (e.g. ceramide) increases HRK levels within 6 h and results in apoptotic cell death within 24 h [48].

We propose the following model to explain the role of AG 205-induced HRK expression in regulating human granulosa/luteal cell survival. In healthy human granulosa/luteal cells higher molecular weight forms of PGRMC1 (i.e. dimers/oligomers) are present. These PGRMC1 dimers/oligomers likely are responsible for suppressing *Hrk* transcription through an unknown mechanism, which ultimately maintains HRK at a very low level. This would allow BCL2 like proteins and MCL1 to bind BAX and BAK thereby preventing their activation and capacity to form channels through which cytochrome c can transit from the mitochondria. AG 205 disrupts the PGRMC1 dimers/oligomers and attenuates PGRMC1's biological action in part by increasing *Hrk* expression. HRK is a transmembrane protein that often resides within the outer membrane of the mitochondria [48, 49]. As HRK is a BH-3 only protein, it can bind to the BCL2 family members (e.g. MCL-1; BCL2 like) [49, 50]. HRK interactions with the BCL2-like proteins are very stable [51], which likely disrupts the interactions between BCL2-like protein/MCL1 and BAX or BAK. This would allow BAX and BAK to be activated and form oligomeric channels through which cytochrome c can translocate from the mitochondria to the cytoplasm [49, 50]. The proposed increase in the formation of the BAX and BAK oligmeric channels within the mitochondria would account for the observed dispersed distribution of cytochrome c throughout the cytoplasm of AG 205-treated cells. However, it is important to appreciate that the putative increase in cytoplasmic levels of cytochrome c is not sufficient to activate the caspase cascade but likely makes the cells more sensitive to an apoptotic stimulus and less responsive to P4's anti-apoptotic action. This model is presented to provide a rationale for future studies on identifying a detailed mechanism through which PGRMC1 signaling preserves the viability of human granulosa and luteal cells.

The present in vitro studies suggest that PGRMC1 signaling suppresses the expression of *Hrk*. This would be consistent with PGRMC1 regulation of HRK being part of its mechanism

that regulates the viability of human granulosa/luteal cells. However, additional genetic-based studies are required to establish a cause and effect relationship between PGRMC1 and the regulation of HRK. In addition these in vitro studies have limitations in their ability to predict a functional role for HRK in regulating ovarian function in vivo. However, environmental stressors like polycyclic aromatic hydrocarbons (PAH) induce *Hrk* expression in the granulosa cells of primordial and primary ovarian follicles [52, 53]. This increase in *Hrk* mRNA is associated with the demise of this population of small follicles and subsequent loss of fertility [53]. In addition the ability of PAH to diminish the primordial/primary follicle population is not observed in HRK knockout mice [53]. These in vivo studies add credence to our hypothesis that PGRMC1 functions to suppress HRK as part of its mechanism to preserve human granulosa and luteal cell viability and is consistent with previous work indicating that point and deletion mutations in PGRMC1 are associated with premature ovarian failure in women [54].

In summary, the present studies reveal that AG 205 interferes with the formation of the higher molecular weight forms of PGRMC1. These higher molecular weight forms are known to localize to the nucleus [26, 30] and likely account for PGRMC1's ability to suppress the expression of genes involved in apoptosis [26, 30] including the BH-3 only protein HRK. We further hypothesize that PGRMC1's ability to suppress HRK is part of the mechanism through which P4-PGRMC1 signaling preserves the viability of human granulosa/luteal cells. However, more genetic-based studies are required to prove this hypothesis.

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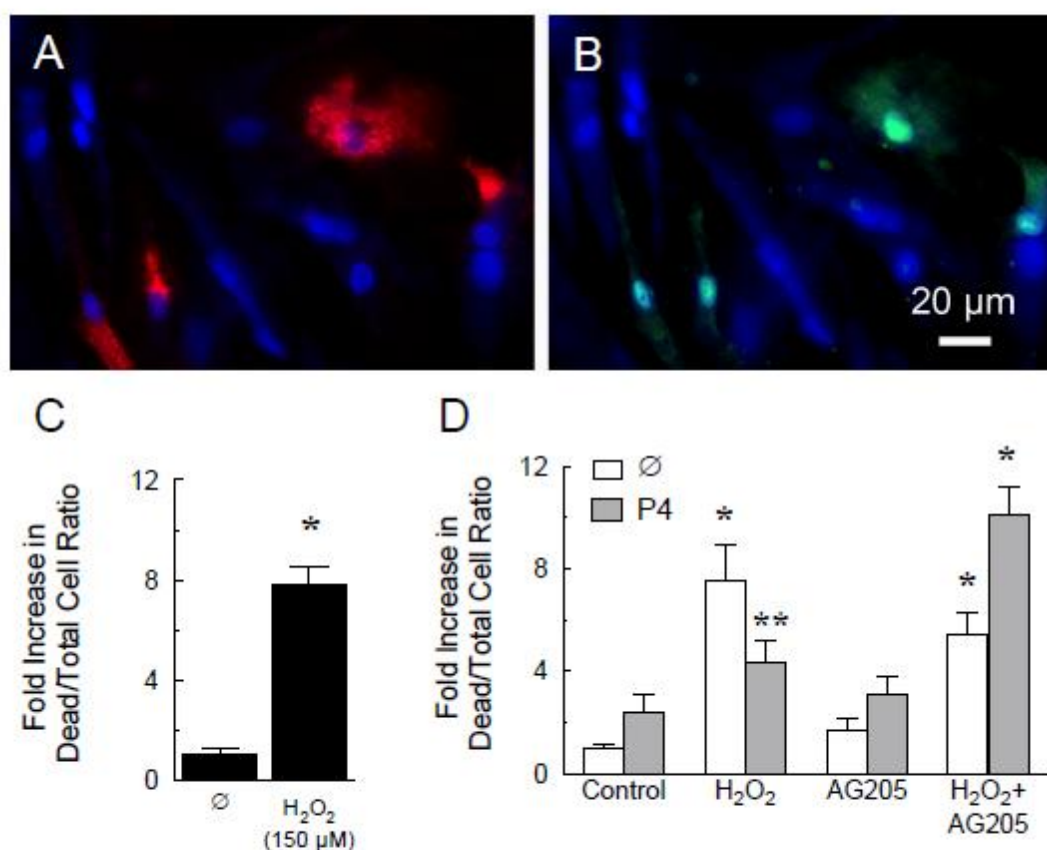
Figure 1. The effect of hydrogen peroxide (H_2O_2; 150 µM), Progesterone (P4, 1 µM) and the PGRMC1 antagonist, AG 205 (50 µM) on the viability of human granulosa/luteal cells. The cells were exposed to H_2O_2 and co-stained for Annexin-V-CY3 (red) **(A)** and 488 dead cell stain (green) **(B)** and the nuclei counter stained with DAPI (blue). The effect of H_2O_2 on the ratio of

dead to total cells is shown in **C** (n= 8 per group collected from two separate experiments). The

effect of P4 and AG 205 on H_2O_2-induced cell death is shown in **D (**n= 16 per group collected

from two separate experiments). In this and the other figures, the values are expressed as a

mean ± standard error. * indicates a value greater than control; ** indicates a value that is

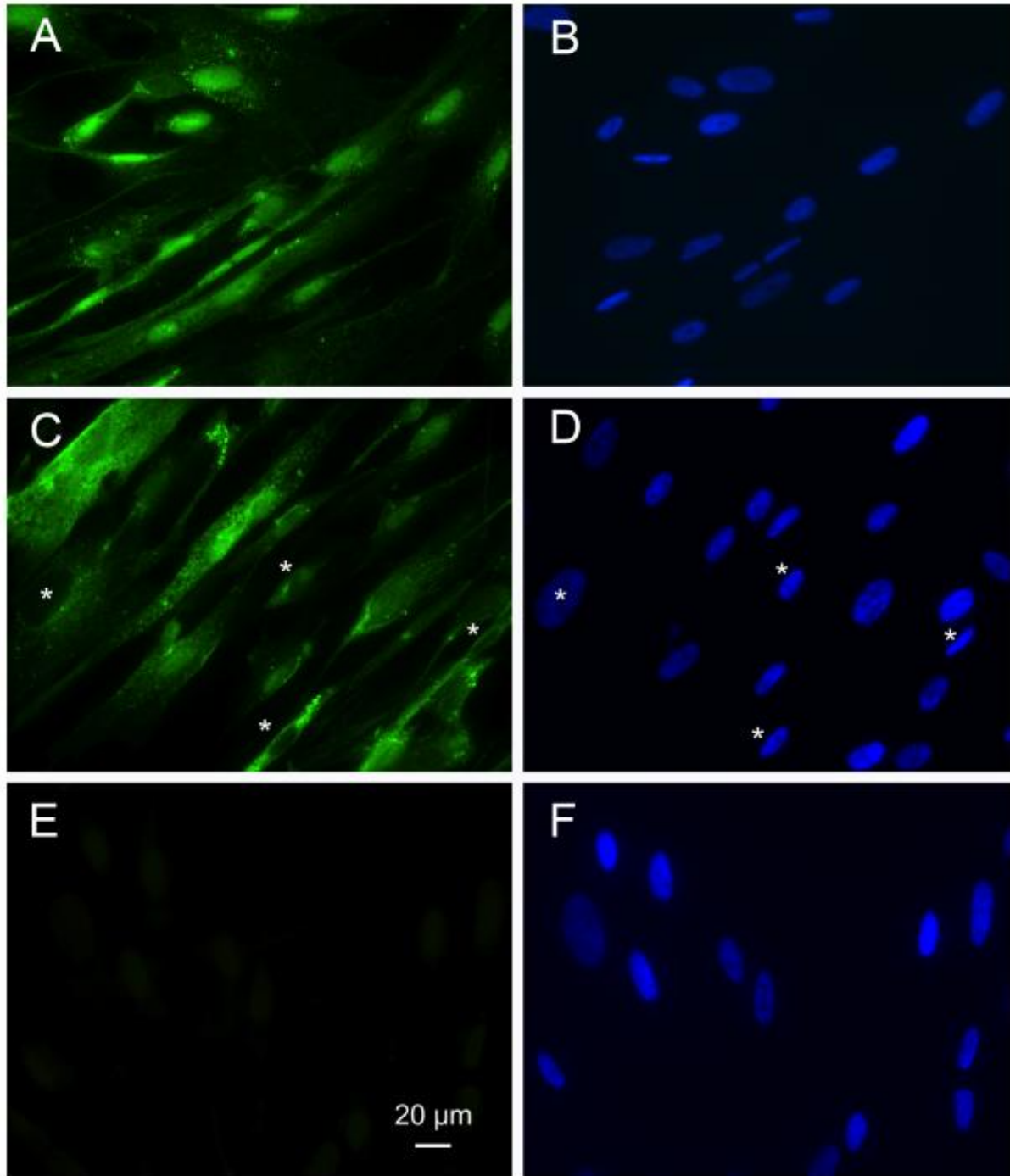greater than control but less than that associated with H_2O_2 treatment ($p < 0.05$).

Figure 2. The effect of DMSO **(A)** or AG 205 **(C)** on the cellular localization of PGRMC1 (green). The * mark nuclei devoid of PGRMC1 staining. Images of DAPI-stained nuclei corresponding to the cells shown in **A** and **B** are shown in **C** and **D**, respectively. Cells stained

in the absence of PGRMC1 antibody are shown in **E** with the corresponding DAPI-stained cells

shown in **F**. Images are representative images from 3 experiments.
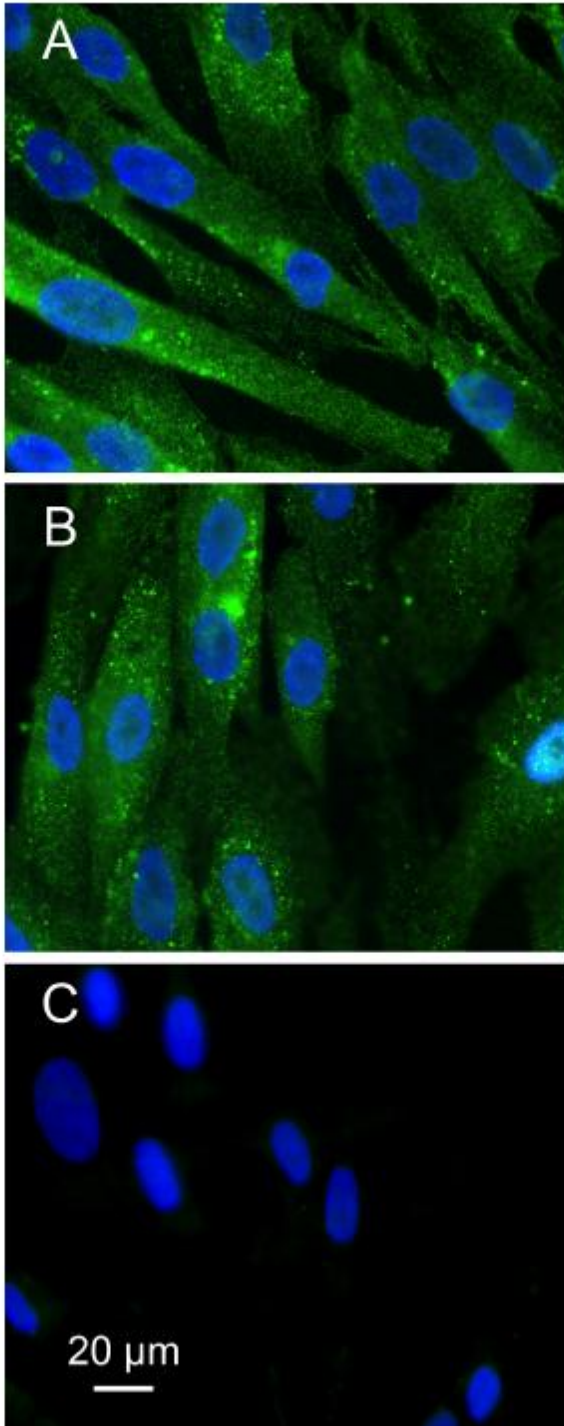
Figure 3. The effect of DMSO **(A)** or AG 205 **(B)** on the cellular localization of PGRMC2.

Merged images reveal PGRMC2 (green) in the cytoplasm, while PGRMC2 is not detected in the

nuclei (blue). A negative control is shown in **C.** Images are representative images from 3

experiments.
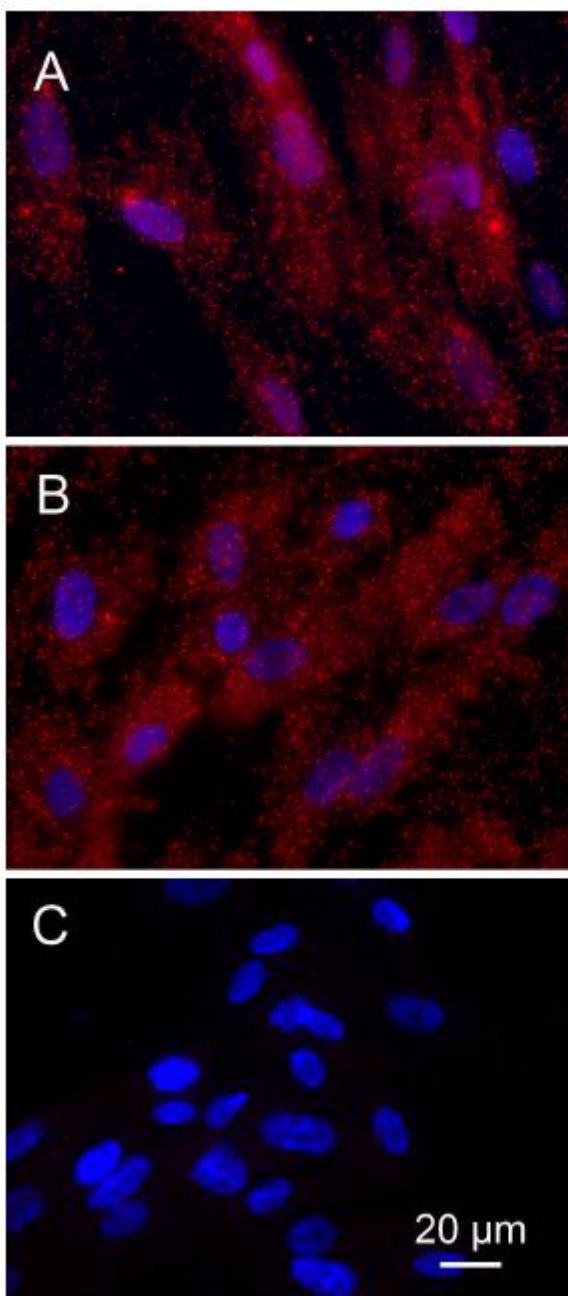
Figure 4. The effect of DMSO **(A)** or AG 205 **(B)** on the interaction between PGRMC1 and

PGRMC2 as reveled by the in situ proximity ligation assay (red dots). Nuclei are stained with

DAPI. A negative control is shown in **C**. Images are representative images from 3 experiments.
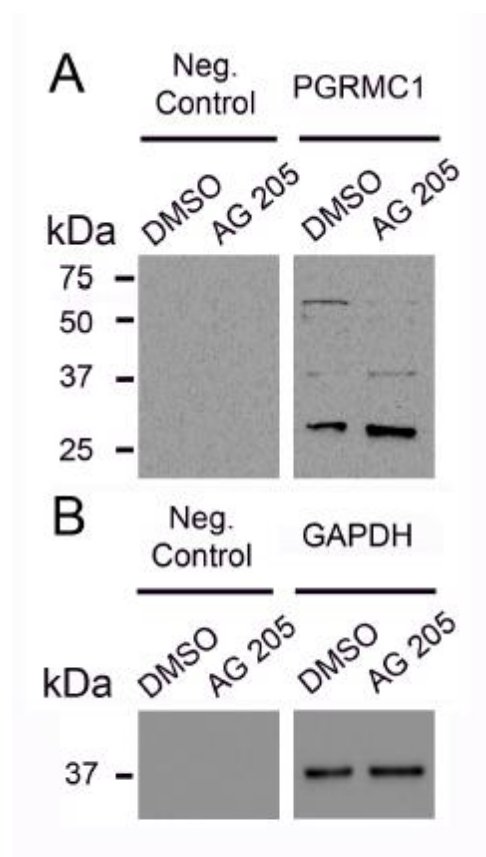
Figure 5. The effect of DMSO or AG 205 on the different molecular weight forms of PGRMC1

as assessed by Western blot **(A)**. A Western blot of GAPDH is shown in **B**. Representative

blots obtained from 2 experiments are shown.
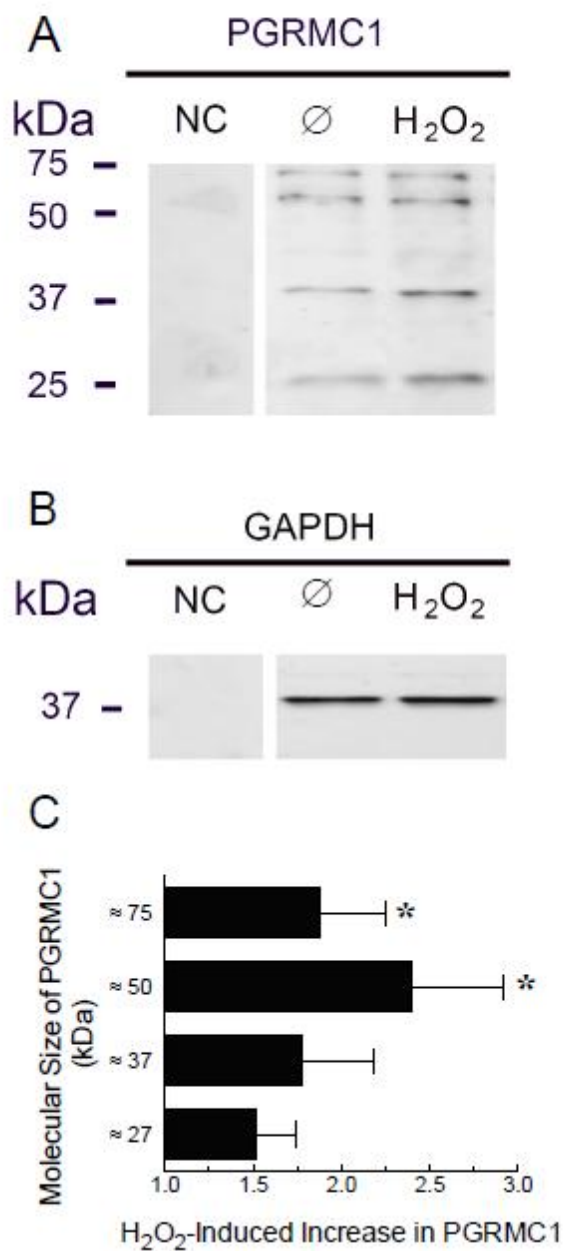
Figure 6. The effect of H_2O_2 on the relative levels of the different molecular weight forms of PGRMC1 as assessed by Western blot (right side of **A**). A negative control (NC) is shown on the left side of **A**. **B** is a Western blot illustrating that GAPDH levels are not affected by H_2O_2. The percent increase in the levels of different molecular weight forms of PGRMC1 over control is shown in **C** (n = 4 from 4 experiments).
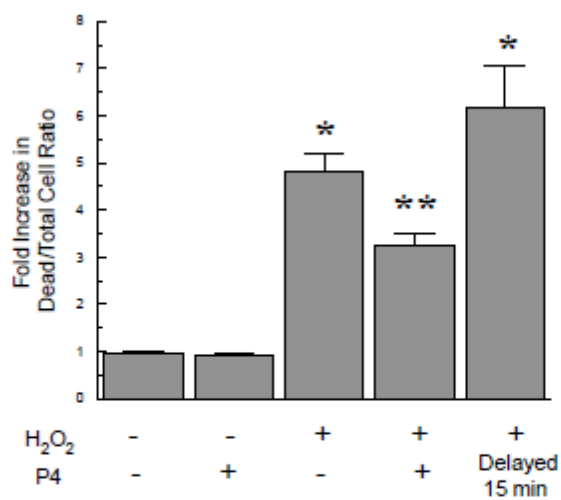
Figure 7. The time-dependent effect of P4 supplementation on H_2O_2-cell death. In this study P4 was administered either simultaneously with H_2O_2 or 15 min after H_2O_2 (n = 22-28 per group from 4 experiments).
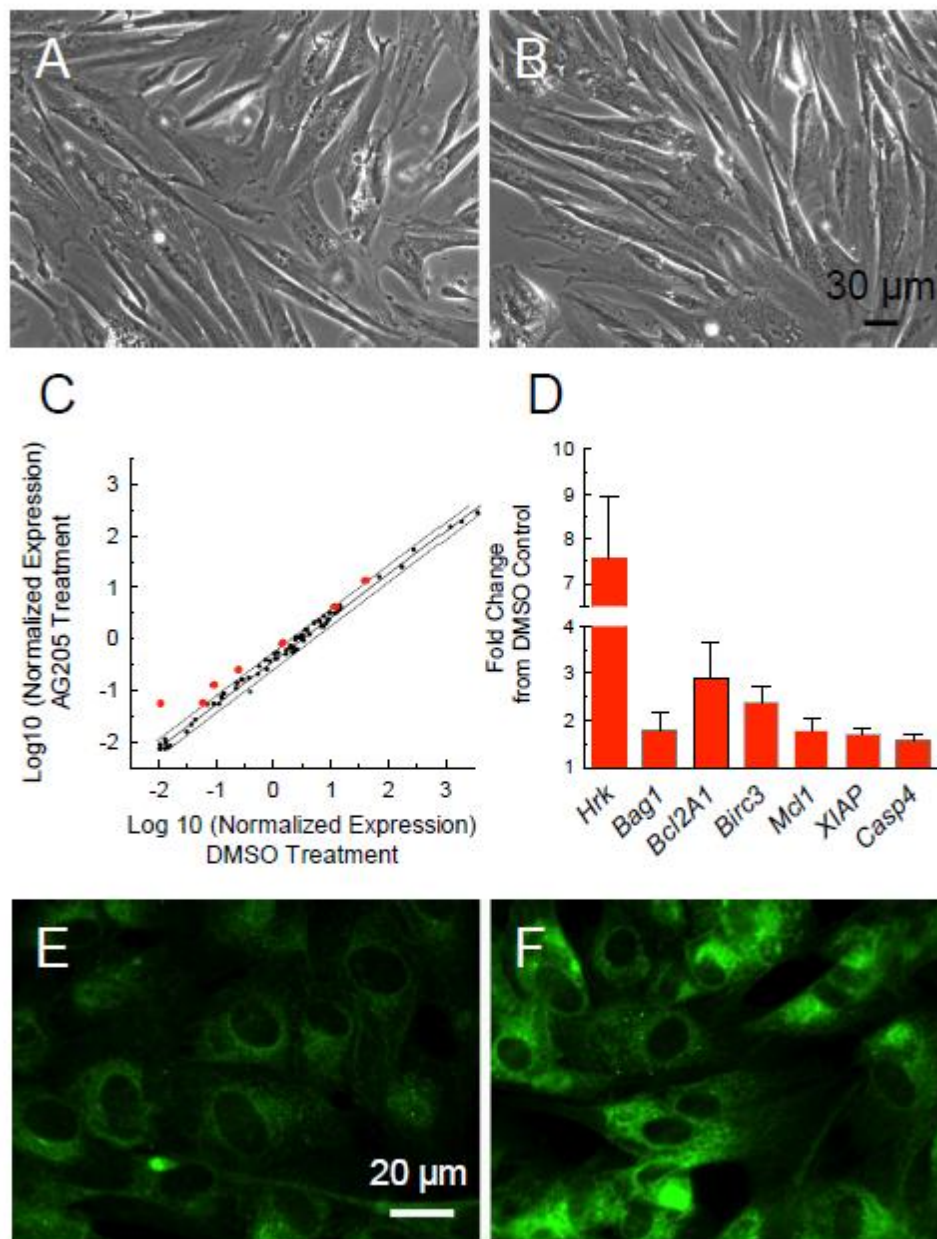
Figure 8. The effect of DMSO **(A)** or AG 205 **(B)** on the morphology of human granulosa/luteal cells as observed by phase microscopy. The relative change in mRNA levels that encode various apoptosis-related genes is shown in **C**. The mean mRNA of each gene is shown as a dot. The means are also fitted to a line with the upper and lower lines representing a ± 1.5 fold change from the means. The red dots represent mRNA levels that are significantly different (n = 5; p < 0.05). The fold change from the DMSO control in these mRNAs is shown in **D**. All

values in **D** are significantly different from DMSO control ($p < 0.05$). The effect of DMSO or AG

205 on the localization and relative expression of HRK as illustrated by intensity of the green

immunofluorescence is shown in **E** and **F**, respectively.
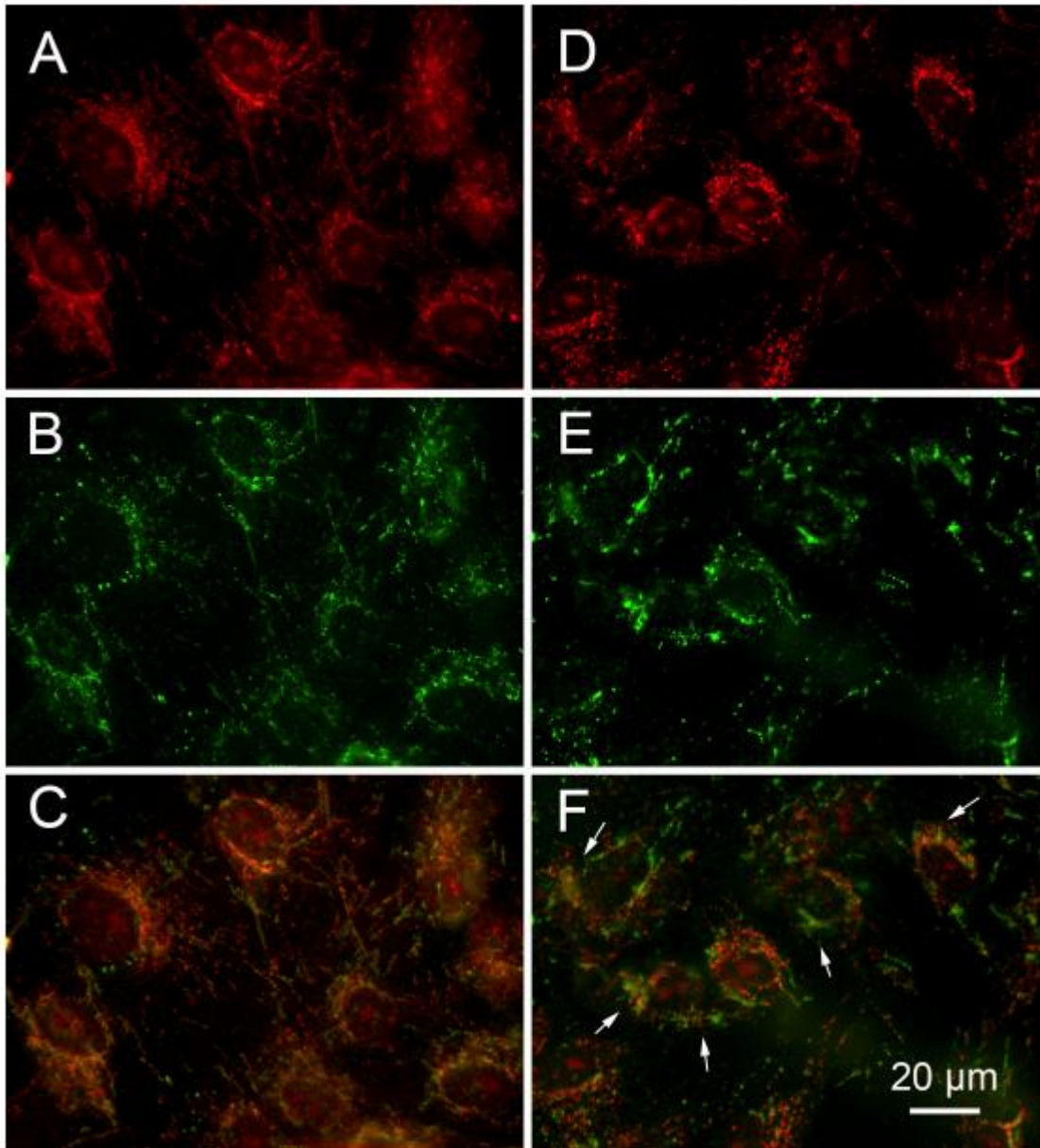

Figure 9. The effect of DMSO (A,B and C) or AG 205 (D,E, and F) on the localization of

cytochrome c (B,E green) in relationship to mitochondria (A, D red). Merged images are shown

in C and F. Note that areas with orange staining reveal areas in which cytochrome c and

mitochondria co-localize. Comparison of the merged images reveal that in the DMSO treated

cells virtually all the cytochrome c co-localizes with the mitochondria, while much of the

cytochrome c does not correspond to the localization of the mitochondrial as reflected in the

large amount of green fluorescence in panel F. White arrows point to areas of cytochrome c

staining not co-localizing to mitochondria. In the negative controls for cytochrome c, the green

fluorescence was essentially non-detectable (note shown) . Images are representative images
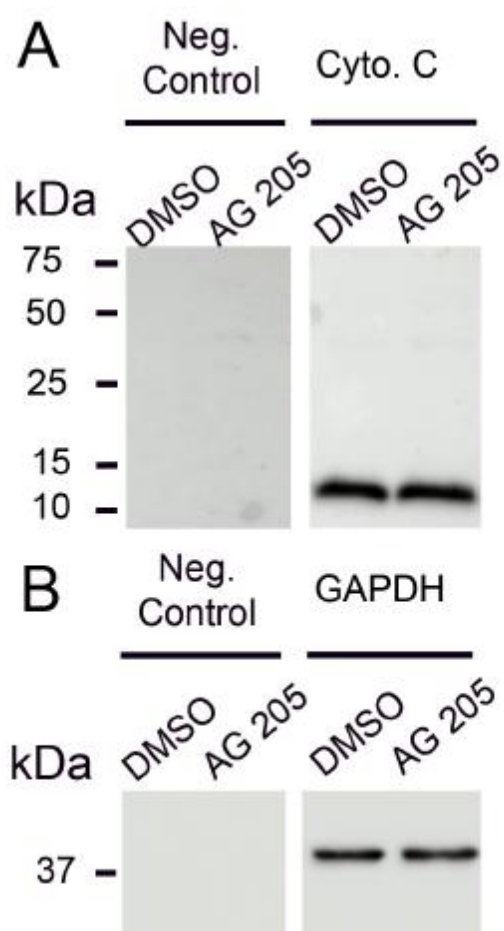
from 3 experiments.

Figure 10. The levels of cytochrome c (Cyto. C) as shown by Western blots of whole cell

lysates collected after treatment with DMSO or AG 205 **(A)**. GAPDH levels from these lysates

are shown in **B.** Negative controls are shown at the left of each panel. Representative blots obtained from 3 experiments.

Supplemental Figure 1. The effect of DMSO or AG 205 on the localization and relative expression of HRK as illustrated by intensity of the green immunofluorescence is shown in **A** and **B**, respectively. A negative control is shown in **C.**

Supplemental Figure 2. The effect of PGRMC2 siRNA treatment on the expression of PGRMC2 as assessed by immunocytochemistry. PGRMC2 is shown in red while the DAPI-stained nuclei are shown in blue.