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Prohibitin (PHB) acts as a potent survival factor against ceramide induced apoptosis in rat granulosa cells*

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Abstract

Aim—Ceramide is a key factor in inducing germ cell apoptosis by translocating from cumulus cells into the adjacent oocyte and lipid rafts through gap junctions. Therefore studies designed to elucidate the mechanistic pathways in ceramide induced granulosa cell (GC) apoptosis and follicular atresia may potentially lead to the development of novel lipid-based therapeutic strategies that will prevent infertility and premature menopause associated with chemo and/or radiation therapy in female cancer patients. Our previous studies have shown that Prohibitin (PHB) is intimately involved in GCs differentiation, atresia, and luteolysis.

Main methods—In the present study, we have examined the functional effects of loss-/gain-of-function of PHB using adenoviral technology in delaying apoptosis induced by the physiological ligand ceramide in rat GCs.

Key findings—Under these experimental conditions, exogenous ceramide C-8 (50 μ M) augmented the expression of mitochondrial PHB and subsequently cause the physical destruction of GC by the release of mitochondrial cytochrome c and activation of caspase-3. In further studies, silencing of PHB expression by adenoviral small interfering RNA (shRNA) sensitized GCs to ceramide C8-induce apoptosis. In contrast, adenovirus (Ad) directed overexpression of PHB in GCs resulted in increased PHB content in mitochondria and delayed the onset of ceramide induced apoptosis in the infected GCs.

Significance—Taken together, these results provide novel evidences that a critical level of PHB expression within the mitochondria plays a key intra-molecular role in GC fate by mediating the inhibition of apoptosis and may therefore, contribute significantly to ceramide induced follicular atresia.

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Keywords

prohibitin; granulosa cells; apoptosis; mitochondria; ceramide

Introduction

Mitochondrial events that include permeability changes, generation of reactive oxygen species, release of cytochrome c and other proapoptotic cofactors that are needed for activation of effector caspases play prominent roles in mediating apoptotic signaling. Additionally, changes in mitochondrial morphology such as fragmentation and cristae remodeling that were previously overlooked are now known to be fundamental to mitochondrial mediated apoptosis in mammalian cells (Cereghetti and Scorrano, 2006). The emerging paradigm suggests that these different signals converge at specific mitochondrial proteins activating or deactivating them prior to apoptosis. In turn, these proteins orchestrate changes in mitochondrial function that facilitate cytochrome c release and progression of the apoptotic cascade. In parallel, however, there are several survival molecules that counteract the activities of these proapoptotic signals (Hussein, 2005; Craig et al., 2007).

Prohibitin has been identified as a molecular factor that can mediate antiapoptotic signals and is essential for mitochondrial function (Mishra et al., 2005). Prohibitin is a member of a highly conserved family of proteins that are thought to play major roles in cell cycle control, differentiation, senescence, and apoptosis (Mishra et al., 2005). To date, there are at least two homologous prohibitin proteins that exist in eukaryotic cells, PHB (prohibitin, b-cell associated protein 32) and REA (repressor of estrogen receptor activity, prohibitin 2, b-cell associated protein 37, prohibition) (Kurtev et al., 2004). A growing body of evidence has implicated a role for PHB in mitochondrial structure, function and inheritance (Nijtmans et al., 2000; Sutovsky et al., 2000; Thompson et al., 1999, 2001; Chowdhury et al., 2007). Both rat and human PHB possess a short transmembrane helix near their N-termini that may be integrated into the mitochondrial membranes (Mishra et al., 2005). Previously, studies performed in our laboratory (Thompson et al., 1997; Chowdhury et al., 2011) identified and characterized PHB in rat GCs isolated from preantral and early antral follicles. We have shown that PHB expression in ovarian tissue is age- and stage- regulated, and that PHB is predominantly localized to the inner mitochondrial membrane of rat GCs (Thompson et al., 2001, 2004). These studies suggest that PHB plays a critical regulatory role in ovarian GC development. More recently, we demonstrated that the expression levels of the 30-kDa Phb protein increases during gonadotrophin-induced GCs differentiation. Moreover, PHB levels also increase in rat GCs during activated apoptosis (Thompson et al., 2001, 2004; Chowdhury et al., 2007). In further studies, infection of GCs with a PHB adenoviral construct that resulted in overexpression of PHB markedly attenuated the ability of staurosporine (STS) and serum starved induction of apoptosis that occurs via the intrinsic apoptotic pathway (Chowdhury et al., 2007).

These experimental observations support our hypothesis that PHB plays a key role in maintaining mitochondrial integrity and delaying apoptosis in GCs. We now know that GCs apoptosis is an important event in follicular atresia (Matsuda-Minehata et al., 2006). However, our understanding of the mechanisms that regulates GCs apoptosis in follicular atresia is limited. In this study we have used ceramide to induce apoptosis in GCs based on its physiological role as a critically important bioactive second messenger involved in mediating cellular differentiation, proliferation, apoptosis and senescence (Pandey et al. 2006). Ceramide biosynthesis is initiated by hydrolysis of phospholipids (sphingomyelin) localized to the outer leaflet of the plasma membrane, which is now recognized as an important pathway of signal transduction. Once ceramide is formed, it induces

mitochondrial translocation of Bax which in turn causes an increase in Bax to Bcl-xl ratio resulting in the release of cytochrome c and has been implicated as an important mediator of cell death by activation of the caspases (Obeid et al., 1993; Pandey et al. 2006). In support of this observation, earlier studies have demonstrated that a ceramide induced dose-dependent apoptosis occurs in early antral follicle (Kaipia et al., 1996) and in GCs from a number of different species including rat (Kim et al., 1999), mice (Kim et al., 1999) and hen (Witty et al., 1996) or in response to pathological insult (Morita et al., 2000) by rapidly decreasing the transmembrane potential of the mitochondria, resulting in activation of the classical intrinsic caspase cascade/pathway (Venavle et al., 1995; Gomez-Munoz, 1998; Zao et al., 2004; Chowdhury et al., 2006, 2008). Moreover, ceramide and acid sphingomyelinase (ASMase; an enzyme that generate the ceramide) plays a central role in the age-related acceleration of apoptosis in the female germline (Bezombes et al., 2004; Perez et al., 2005; Takai et al., 2003). Furthermore, TNF α induced sphingomyelin hydrolysis also occurs with the subsequent increase in apoptosis in cultured GCs (Santana et al., 1995). Currently, there are no studies known regarding ceramide dependent PHB expression and modulation of GCs apoptosis. The aim of the present study therefore was to determine whether the gain or loss of PHB function impact ceramide induced apoptosis in a rat ovarian GC model.

Materials and Methods

Granulosa Cell (GC) Cultures

Primary GCs were isolated from immature (23 days old) Sprague-Dawley rat ovaries as previously described (Thompson et al., 1997). Ovaries were collected into serum-free medium (4F), which consists of 15 mM HEPES, pH 7.4, Dulbecco's modified Eagles's medium/F-12 with transferrin (5 μ g/ml), human insulin (2 μ g/ml), hydrocortisone (40 ng/ml), and antibiotics. After incubating the ovaries at 37°C in 4F medium containing 0.5 M sucrose and 10 mM EGTA for 30 min, the ovaries were washed in fresh 4F medium. GCs were collected from the ovaries by puncturing follicles with a 25-gauge hypodermic needle, and cells were dispensed into 4F medium supplemented with 10% FBS (GIBCO BRL, Grand Island, NY), and incubated in a humidified atmosphere of 5% CO₂ at 37°C. All animal care handling procedures in the present study were approved by the Institutional Animal Care and Use committee in accordance with the guidelines of the National Institutes of Health (NIH) and the U.S. Department of Agriculture (USDA).

Induction of Apoptosis

Culturing GCs in serum-free medium in the presence of N-Octanoylsphingosine (ceramide C-8; Biomol, Plymouth Meeting, PA) have been shown to induce apoptosis (Witty et al., 1996). For ceramide C-8 concentration and time response studies, GCs were cultured for 24 h in the presence of different concentrations (25, 50 and 100 μ M) of ceramide C-8 at different times (0, 2, 4, 8, 12, 16 and 24 hrs). Following ceramide C-8 treatment the percentage of apoptosis was determined by nuclear staining with Hoechst 33248 stain (12.5ng / ml; Sigma) as described by Sasaki et al., (2000) or by using the annexin V Vybrant apoptosis assay kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR) and caspase 3 activity assay as described below. Total cells (attached and detached) were labeled with annexin V and positive cells were counted for each field examined (at least 10 fields per plate). The percentage of annexin V positive cells was calculated as the number of annexin V positive cells divided by the total number of cells. The assay was repeated on three GC preparations. The data were plotted as percent apoptotic cells against time in hours.

Caspase Enzymatic Activity

Caspase-3 activity was measured using a colorimetric assay kit (CaspACE-colorimetric, Promega, Madison, WI, USA). After apoptotic induction of GCs with 50 μ M ceramide C-8, cells were pelleted by centrifugation at 200 xg for 10 min at 4°C, washed with ice cold NaCl/Pi, and resuspended in the CaspACE cell lysis buffer (1.0×10^8 cells / ml). The cell lysates were incubated on ice for 15min and centrifuged at 15,000 xg at 4°C for 20min. The supernatant was collected and stored at -80°C. The protein concentration was measured in the resulting supernatant using Bradford protein assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA) with a BSA reference standard. The caspase-3 assay was performed in 96 well plates. Fifty microgram of extract protein was added together with the DEVD-p-nitroaniline substrate. The plate was incubated at 37°C and the p-Nitroaniline released by active caspase was measured at 405nm using a microplate reader (Molecular devices, Sunnyvale CA). A p-nitroaniline calibration curve was obtained using a p-nitroaniline standard diluted in dimethylsulphoxide (DMSO). Caspase-3 activity was calculated in picomol/h/ μ g-protein and plotted on the graph as % of control. Control was taken as 100% in each group.

Isolation of S-100 fraction and mitochondria

S-100 (cytosolic) fractions and mitochondria were prepared as described (Thompson et al., 2001), with modification. Briefly, primary GCs were harvested in PBS at 4°C. Cell pellets were resuspended in 5 vol buffer A (20mM HEPES-KOH (pH7.5), 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 0.1mM phenylmethylsulphonylfluoride, 1% aprotinin, 10 μ g/ml leupeptin, 1 μ g / ml pepstain A, and 250mM sucrose) and held at 4°C for 10 min. Cells were homogenized, and nuclei and cellular debris were removed by centrifugation at 500x g for 10 min at 4°C. Mitochondria were collected from the supernatant by centrifugation at 10,000x g for 15min at 4°C, resuspended in buffer A, and held at -80°C. Cytosolic proteins were extracted by centrifugation of the mitochondrial supernatant at 100,000X g for 1h at 4°C. Protein levels of cellular fractions were analyzed by Western blot.

Generation of Recombinant Adenoviral Plasmid Vectors

The prohibitin gene PHB was amplified by PCR using rat ovarian cDNA template. The specific primers used were: (5'-GTCGACCATGGCTGCCAAAGT-3', 5'-AAGCTTGGGGTGGGAGCAGAAGGAA-3') containing *SalI* and *HindIII* sites (underlined), respectively. The PCR product was subcloned into a pGEM-T-Easy vector (Promega, Madison, WI) to create pGEM-T-Easy-PHB. The plasmids pGEM-T-Easy-PHB and pAdTrack-cytomegalovirus [CMV; AdEasy system and a CMV promoter] were digested using *SalI* and *HindIII* and religated, resulting in pAdTrack-CMV-PHB. The AdEasy system was used for generation of the recombinant adenovirus. The resultant pAdTrack-CMV-PHB encoded a PHB gene under the control of a CMV promoter. This plasmid pAdTrack-CMV-PHB was co-transformed into electro-competent BJ5183 bacteria with pAdEasy-1 (containing the viral backbone) and selected on Kanamycin LB plates. The plasmid in the bacteria was amplified and purified using a plasmid maxiprep system (Qiagen, Valencia, CA). The complete adenovector was linearized and used for transfection of Ad293 cells (human embryonic kidney cell line), where viral particles were further amplified, purified, and titered according to the manufacturer instruction (Somers et al., 1999; Chowdhury et al., 2007). An adenovirus (Ad) was used as a control.

The shRNA cassettes specifically targeting the nucleotide of PHB were designed through shRNA Target Finder (GenScript, Scotch Plains, NJ). Oligonucleotides bearing 100% identity among available cDNA sequences of target genes from human were selected by BLAST homology search. Three regions of the PHB transcript were selected for shRNA

targets. PCR reactions were performed using a single reverse primer to generate a shRNA expression cassette (SEC) consisting of a U6 promoter and a hairpin shRNA cassette plus terminator. The complementary shRNA oligonucleotides were cloned into the pRNA-U6.1/Neo vector. This process involved annealing the oligonucleotides and ligating the double stranded nucleotide in the above stated vector. Briefly, purified oligonucleotides were resuspended in TE buffer to a concentration of 100 μ M. The oligos (top and bottom strand) were mixed in a 1:1 ratio, resulting in a 50 μ M concentration of double stranded oligonucleotides. The mixture was heated (95°C; 30 sec) to remove secondary structures, allow for the internal hairpin of each oligonucleotide to be disrupted, and to promote intermolecular annealing. This was followed by sequential heating (72, 37, and 25 °C; 2 min each) and the mixture was stored on ice.

The annealed oligonucleotides were then ligated into a shuttle vector, pAdTrack-CMV. The oligonucleotides were diluted using TE buffer to a final concentration of 0.5 IM. Ligation reactions were performed with both experimental and negative control oligonucleotides, followed by incubation at room temperature for ~3 h. The resultant plasmid was linearized by digesting with restriction endonuclease PmeI, and subsequently cotransformed into *E. coli* cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinants are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearized recombinant plasmid is transfected into Ad packaging cell lines, Ad293 cells for production of viral lysates.

Adenoviral (Ad) infection of Granulosa cells (GCs)

GCs were grown on 6-well culture dish in 4F media supplemented with 10% FBS. Subsequently, medium was removed and cells were washed twice with Opti-MEM (antibiotics-free) and infected with Ad vector at a multiplicity of infection (MOI) of 1, 5 or 10 plaque forming units per cell (PFU/cell). Medium was replaced with 4F medium supplemented with 10% FBS after 2h of incubation at 37°C with occasional rocking. GCs showed 95- to 100% expression at 10 PFU/cell (data not shown). Twenty-four hours after exposure to adenoviruses, the media was removed and replaced with 4F medium without FBS also in the presence and absence of 50 μ M ceramide C8.

Western Blot Analysis

GC fractionation (mitochondria, nucleus and cytosol) protein extracts obtained from different treatment conditions were subjected to one-dimensional gel electrophoresis. The procedures used for one-dimensional gel electrophoresis, protein transfer, and blotting has been described previously (Thompson et al., 2001). Primary antibodies used were rabbit polyclonal prohibitin (PHB) (1:1000; Neomarks, Fremont, CA), mouse monoclonal cleaved caspase 3 or cytochrome c (1:1000; Cell Signaling, Beverly, MA) and α -tubulin or porine (1:1000; Neomarks, Fremont, CA). Membranes were incubated with the appropriate secondary antibody for 2 h at room temperature, and antibody binding was detected by chemiluminescence (Pierce, Rockford, IL). Results of representative chemiluminescence were scanned and densitometrically analyzed using a Power Macintosh Computer (G3; Apple Computer Inc., Cupertino, CA) equipped with a ScanJet 6100C Scanner (Hololett-Packard Co., Greeley, CO). Quantification of the scanned images was performed according to the NIH Image version-1.61 software (National Institute of Health, Bethesda, MD).

Statistical analysis

All experiments were replicated a minimum of three times, unless otherwise stated. Data are expressed as mean $\pm$ SEM of three experiments. Statistical analysis was performed by one-way ANOVA using SPSS version 11.0 software (SPSS, Chicago, IL). Multiple comparisons were done by Newman-Keuls' test. Differences were considered significant at $P \leq 0.05$.

Results

Ceramide C-8 affects PHB expression and induced mitochondrial cytochrome c release during apoptosis in undifferentiated granulosa cells (GCs)

We first determined the optimum concentration of ceramide for the induction of apoptosis in GCs. Undifferentiated primary GCs were grown to 95-100% confluence and treated with different doses (25, 50 and 100 μ M) for 24h in serum free media. As shown in figure 1, ceramide C8 potently and significantly caused apoptotic cell death in a dose and time-dependent manner at all doses ($p < 0.05$; Newman-keuls' test). Indeed, there was a 100% apoptotic cell death versus control cells in response to the two higher doses (50 and 100 μ M respectively) of ceramide ($p < 0.05$). Based on these results we selected 50 μ M dose for all other experimental studies. Likewise, caspase-3 activity was markedly increased at 12 to 24 h after ceramide C8 (50 μ M) treatment of GCs (Fig 2B). To determine the effects of ceramide C8 on the level of PHB expression during the onset of apoptosis in GCs, cultured cells were exposed to ceramide C8 at the concentration of 50 μ M dose for the indicated time (0-24h) period. At different time intervals of ceramide C8 (50 μ M) treatment, the cytosolic and mitochondrial extractions were analyzed for the determination of PHB protein content, the release of the proapoptotic mitochondrial proteins cytochrome *c* and procaspase-3 cleavage. As shown in figure 2C and 2D, Western blot analyses revealed that Phb protein content increased during the initial 12h of ceramide C8 treatment. However, with the release of the proapoptotic mitochondrial proteins cytochrome *c* to the cytosol and the cleavage of procaspase 3, we observed a decreased in the expression levels of Phb (Fig. 2C, D).

Adenoviral-mediated knockdown of Phb sensitized granulosa cells to ceramide C8-induced apoptosis

To determine the efficiency and specificity of adenoviral shRNA (AdshRNA) in reducing PHB gene expression, Western blot analysis was performed in GCs infected with equal viral titers (achieved by adding the appropriate amount of empty adenoviral vectors, Ad-scrambled). Dose and time dependent knock-down of PHB in infected GCs was determined from separate experiments using immunofluorescence microscopy and Western blotting techniques. Infection with empty vector (Ad-scrambled) alone did not show any change in PHB levels (data not shown). GCs were infected with 10 MOI of Ad-scrambled (control) and adenoviral shPHB (AdshPHB). Forty-eight hours after AdshPHB infection, we determine the effects of PHB down-regulation on ceramide C8-induced apoptosis. GCs were treated for 0, 2, 4, 8 and 12 hr respectively with ceramide C8 (50 μ M) 48 h post-AdshPHB or Ad-scrambled infection. The nuclear morphologic changes occurring in undifferentiated GCs in the presence or absence of adenovirus directed knock-down of PHB with or without ceramide C8 treatment were assessed by comparing chromatin condensation and fragmentation into apoptotic bodies by using nuclear stain Hoechst 33248. As shown in figure 3A, knock-down of PHB enhanced apoptosis in a duration dependent manner in the presence of ceramide C8 when compared to Ad-scrambled infected cells ($p < 0.05$; Newman-keuls' test).

In further studies, we examined whether knock-down of PHB resulted enhanced the processing of procaspase-3 to the active enzyme. As shown in figure 3B, knock-down of PHB increased caspases-3 activity significantly ($P \leq 0.05$) in a time dependent manner compared to Ad-scramble virus infected cells. There was a two-three fold difference in caspase-3 activity in cells infected with empty vector vs the AdshPHB treated group in the presence of ceramide C-8 treatment at 2, 6 and 8h ($P \leq 0.05$). Immunofluorescence microscopy was used to evaluate percentage of apoptotic cells as detected by annexin V staining in the AdshPHB and Ad-scramble infected cells that were treated with ceramide C8. GCs cultured with Ad-scrambled showed a significantly lower numbers of annexin V-

positive cells at 8h time point after ceramide C-8 treatment (figure 3C panel c and d), whereas GCs infected with AdshPHB followed by ceramide C-8 treatment showed higher annexin V staining with the classical morphological features of apoptosis including the formation of apoptotic bodies and membrane blebbing (figure 3C, panels g and h).

Then, we examined whether knock-down of PHB inhibited the release of cytochrome c from the mitochondria and processing of procaspase-3 to the active enzyme. Cells were treated with ceramide C8 48h post-AdshPHB infection and then harvested at 0, 2, 4, 8 and 12h. As shown in Fig.3D, Western blot analyses revealed that PHB levels were significantly decreased over the 48h period with the maximum expression level occurring at 8 and 12h when compared to Ad-scrambled control ($P \leq 0.05$). In parallel studies, knock-down or the silencing of the PHB gene markedly enhanced the release of cytochrome c and an earlier procaspase-3 cleavage (8 h) when apoptosis was induced by treatment with ceramide C8 (compare figure 4D). In contrast, no cleaved caspase-3 was observed at 2, 4 and 8hr in Ad-scrambled (control) cells treated ceramide C8.

Adenovirus directed over-expression of PHB inhibits ceramide C8 induced apoptosis in granulosa cells

To test the function of PHB protein during the onset of apoptosis, its over-expression was precociously forced in cultured GCs. This was accomplished using replication-defective recombinant adenoviruses that were previously characterized in our experimental system (Chowdhury et al., 2007). GCs were infected with AdPHB sense cDNA [multiplicity of infection (MOI) of 10] for 24h. Dose and time dependent expression of PHB in infected GCs was determined from separate experiments using immunofluorescence microscopy and Western blotting techniques (Chowdhury et al., 2007). Infection with empty vector alone did not show any change in Phb levels (data not shown). Following infection with adenovirus with or without PHB, GCs were exposed to ceramide C8 at the concentration of 50 μ M for the indicated time period (0-24h) in serum free media. The nuclear morphologic changes occurring in undifferentiated GCs in the presence or absence of adenovirus directed expression of PHB with or without ceramide C8 treatment were assessed by comparing chromatin condensation and fragmentation into apoptotic bodies by using nuclear stain Hoechst 33248. As shown in figure 4A, over expression of PHB delayed apoptosis in a duration dependent manner in the presence of ceramide C8 when compared to controls ($p < 0.05$; Newman-keuls' test).

In further studies, we examined whether over expression of PHB resulted in the inhibition of the processing of procaspase-3 to the active enzyme. In figure 4B, we showed that over expression of PHB significantly delayed caspases-3 activity ($P \leq 0.05$) in a time dependent manner compared to empty vector. There was a two-fold difference in caspase-3 activity comparing cells infected with empty vector vs the AdPHB treated group in the presence of ceramide C-8 treatment at 16 and 24h ($P \leq 0.05$). Immunofluorescence microscopy was used to evaluate percentage of apoptotic cells as detected by annexin V staining in the AdPHB and Ad control infected cells that were treated with ceramide C8. GCs cultured with adenovirus with empty vector (control) showed significant increase in numbers of annexin V-positive cells beyond the 8h time point after ceramide C-8 treatment (figure 4C, panels a and b) whereas GCs infected with AdPHB followed by ceramide C-8 treatment showed no annexin V staining (figure 3C, panels c and d). A higher magnification image (Fig. 4C, inset a) allows better resolution of annexin V labeling observed in the GCs.

Next, we examined whether over expression of PHB inhibited the release of cytochrome c from the mitochondria and processing of procaspase-3 to the active enzyme. Cells were treated with ceramide C8 24h post-AdPHB infection and then harvested at 2, 4, 8, 12, 16 and 24h. As shown in Fig. 3D and 3E, Western blot analyses revealed that PHB levels were

increased over the 24h period with the maximum expression level occurring at 12h when compared to control ($P \leq 0.05$). In parallel studies, over expression of PHB markedly suppressed the release of cytochrome c and the cleavage of procaspase-3 to caspase-3, resulting in very low levels of cytochrome c release and cleaved caspase-3 in the cytosol even after 24h of ceramide treatment (compare figure 4D with 2C).

Discussion

Our current studies are designed to elicit the intricate relationship between ceramide and PHB in GC apoptosis. In this study, we have used an analogue of ceramide; ceramide C-8 to determine whether the gain or loss of PHB function impact ceramide induced apoptosis in a rat ovarian GC model. Using this model of programmed cell death, we demonstrated that PHB did act as a potent survival factor against ceramide C-8 induced apoptosis. Ceramide, a key molecule in the metabolism of sphingolipids, is converted by ceramidase to sphingosine, and phosphorylated by ceramide kinase to form ceramide-1-phosphate (C1P). Ceramide has emerged as a key regulator of a wide spectrum of biological processes including cellular differentiation, proliferation and senescence. Moreover, acid ceramidase (AC); a ceramide-metabolizing enzyme is expressed in human cumulus cells and follicular fluid, and the levels of this enzyme are positively correlated with the quality of human embryos formed in vitro (Eliyahu et al., 2010). Our *in vitro* experiments using isolated GCs from rat follicles demonstrated that exposure of these cells to ceramide C-8 directly activated cytochrome c and the cleavage of procaspase-3 from mitochondrial inter membrane space in a time dependent fashion. This finding is consistent with published reports showing that activation of caspase-3 is the hallmark of the point of no return in apoptotic signaling in mouse and rat ovarian GC (Robles et al., 1999; Chowdhury et al., 2007, 2008). Thus, finding in this study suggests that the ceramide C-8 induced mitochondrial cytochrome c release and activation of caspase 3 is an important event in the apoptosis of GCs (Morita et al., 2000; Perez et al., 2005).

In an effort to determine the role of PHB in ceramide C-8 mediated GCs apoptosis, we investigated the pattern of PHB expression during ceramide C-8 induced apoptosis of undifferentiated GCs. Our results suggest that increasing ceramide C-8 levels increased mitochondrial PHB expression that may be a critical response for the stabilizing GCs by correcting stress induced imbalances. To our knowledge, this result demonstrated for the first time that exogenous ceramide C-8 is strongly associated with mitochondrial PHB expression, cytochrome c release from mitochondria and activation of cytosolic caspase-3 in GC. However, as is evident from our results, ceramide treatment for longer duration actually causes a decrease in PHB expression levels with consequent loss of mitochondrial cytochrome c and activation of caspases-3. This suggests that supraphysiological levels of ceramide C-8 can overwhelm the normal physiological protective effect of PHB thereby allowing the cells to undergo apoptosis unabated (Coats et al., 2001; Nijtmann et al., 2002; Thompson et al., 2001; Artal-Sanz et al., 2003; Chowdhury et al., 2007). Moreover, this indicates a physiological role for differentiated expression pattern of PHB during folliculogenesis (Thompson et al., 2004).

In order to evaluate the role of PHB in ceramide C-8 induced apoptosis, we utilized a recombinant adenovirus vector to constitutively overexpress or knock-down PHB in GCs. We have previously demonstrated that replication-defective adenovirus vectors are able to efficiently direct the expression of recombinant proteins in GCs. Greater than 90% of infected GCs in primary culture exhibited adenovirus expression of the green fluorescent protein reporter gene without any adverse effects on cellular function (Chowdhury et al., 2007). Moreover, previous studies have shown that small interfering RNAs can effectively induce sequence specific post-transcriptional target gene silencing in a number of cancer cell

lines (Bourguignon et al., 2007; Whitehurst et al., 2007). Accordingly, we have used this strategy to knock-down Phb gene expression in GCs to determine the role of PHB in the regulation of ceramide C8 induced apoptosis. Our studies showed that PHB knockdown resulted in an early onset of apoptosis in ceramide C-8 treated GCs, as evidenced by the release of mitochondrial cytochrome c release with the cleavage of procaspase-3 to its active caspase-3 enzymatic form. These results are in agreement with previous reports in Hela cells, yeast and *C. elegans*, suggesting that PHB is an essential mitochondrial protein involved in maintaining mitochondria integrity (Kasashima et al., 2006; Tatsuta et al., 2005; Artal-Sanz et al., 2003).

Furthermore, adenoviral directed overexpression of PHB abrogated ceramide C-8 induced cytochrome c release and the activation of caspase 3 and thereby preserved GC viability. The preservation of cell viability in response to PHB over-expression is also associated with enhanced translocation of Phb into the mitochondria in response to ceramide induced apoptotic signals. This observation is consistent with our previous study (Chowdhury et al., 2007, 2011). Thus, these results have provided further additional support for the role of PHB's as an anti-apoptotic agent in immature rat GCs.

We have proposed the following schematic diagram in Fig. 5 for the protective role that mitochondrial PHB is likely to play in GCs apoptosis. Induction of apoptosis by ceramide C-8, results in increased PHB expression in the mitochondria that directly inhibits the release of cytochrome c from the inter-mitochondrial space resulting in inhibition of the activation of cleaved caspase-3. An alternative mechanism leading to inhibition of caspase-3 after mitochondrial PHB overexpression may, in part, be due to the inhibition of cytochrome c translocation into the cytosol thus protecting the mitochondria from undergoing functional incapacitation. Thus, PHB may have an effect on cellular anti-apoptosis events in the GCs by delaying the activation of the apoptotic pathways.

In summary, these experiments have begun to tease apart the ceramide C-8 induced apoptosis in periovulatory GCs with uncovering a novel role of mitochondrial PHB as a key anti-apoptotic or survival factor in this process. Our present work has further established that the 30-kDa PHB is localized to the mitochondria and has a direct relationship with cytochrome c release and subsequently activation of caspase-3 expression in the cytosol in response to apoptosis in GCs. Further, this findings support that over-expression of PHB in GCs may inhibit the rate of oocyte depletion in females prior to menopause through the interrelationships in ceramide levels, ceramide sensitivity, and acid spingomyelinase (ASMase) availability in oocytes of young and aged female, which collectively raise the apoptotic threshold in female germline with advancing maternal age or during follicular atresia. Although the specific pathway involved in PHB mediated GC survival is unknown, it probably involves multiple signaling mechanisms. Thus, PHB may act either as a sensor for mitochondrial integrity and homeostasis and/or as a survival factor that mediates cell fate decisions during cellular differentiation.

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Fig.1. Induction of apoptosis by ceramide C-8 (N-octanoylsphingosine) in the granulosa cells (GCs)

GCs were grown in culture dishes followed by treated with or without ceramide C-8 (25, 50 and 100 μ M) at different doses for 24h in serum free media. Then fixed and stained with Hoechst 33248 to identify nuclei. Data represent the percentage of cells displaying morphological alteration of apoptosis based on quantification of nuclear morphologic changes. At least 250-300 cells were counted for each data point. The bar graph represents the mean $\pm$ SEM of results from three replicate experiments. Significant differences are represented with different alphabets (a, b, c, d and e) at $P \leq 0.05$ in the respective panels. Same alphabets are showing insignificant differences.

Fig.2. Ceramide C-8 induced morphological and biochemical changes in granulosa cells (GCs) GCs were grown in culture dishes and exposed with or without ceramide C-8 (50 μ M) in serum free media for different time intervals (upto 24h). **A. Ceramide C-8 induced morphological changes in GCs.** Live cell images were captured at 400x magnification by a phasecontrast microscope at different time (C -Control, 2, 4, 8, 12, 16, 24 and 24> h) intervals to represent the morphological changes of cells. **B. Ceramide C-8 induced caspase-3 activity in GCs.** Caspase 3 activity in cytosolic protein extracts from granulosa cells treated with or without ceramide C-8 (50 μ M) was measured using the spectrophotometric substrate DEVD-pNA(D). Graphically, activities are represented as % of control groups. All numerical values are represented as mean $\pm$ SEM of three individual experiments (n = 3). The groups with same alphabets are insignificantly different compared with respective parallel control ($P \leq 0.05$). **C. Ceramide C-8 induced prohibitin (PHB) and active caspase-3 expression, and cytochrome c released from mitochondria to cytosol in GCs.** Western blot analyses of protein levels for PHB, active caspase-3 and cytochrome c release from mitochondria to cytosol in GCs induced by ceramide C-8. Twenty five micrograms of protein from ceramide C-8 (50 μ M) treated GCs for different time (0, 2, 4, 8, 12, 16 and 24 h) interval were applied to each lane and analyzed for protein levels for PHB, cleaved caspase-3 and cytochrome c by Western blot analysis. Representative blots were scanned using the NIH Image software program computer-assisted analysis system for quantitative assessment of changes in protein levels. Porin and α -tubulin were used as an internal control for mitochondria and cytosol respectively. The representative blots are used from three individual experiments (n = 3) performed to show the differences in expression pattern in proteins. **D.** The bar graph represents expression of PHB in the mean $\pm$ SEM from three replicate experiments after normalization of data against porin protein. Different alphabets indicate a significant difference ($P < 0.05$) when compared with control.

Fig.3. Effects of silencing the prohibitin (PHB) gene on ceramide C-8 induced apoptosis in granulosa cells (GCs)

GCs were transiently infected with AdshPHB (MOI 10) or Ad-scrambled (MOI 10) for 2 hr and maintained in culture for 48 hr in serum free media. After 48 h, media was replaced with fresh media with or without ceramide C-8 (50 μ M) in serum free media. Apoptosis was determined at different time (0, 2, 4, 8 and 12 h) interval. **A. Percent apoptosis in adenovirus-directed knock-down PHB treated with or without ceramide C-8 (50 μ M) GCs.**

After each treatment cells were fixed and stained with Hoechst 33248 to identify nuclei. Data represent the percentage of cells displaying morphological alteration of apoptosis based on quantification of nuclear morphologic changes. At least 250-300 cells were counted for each data point. The bar graph represents the mean $\pm$ SEM of results from three replicate experiments. Significant differences are represented with different alphabets (a, b) at $P \leq 0.05$ in the respective panels. **B. Caspase-3 activity in adenovirus-directed knock-down PHB treated with or without ceramide C-8 (50 μ M) GCs.** Caspase-3 activity in cytosolic protein extracts from adenovirus directed with or without PHB treated with or without ceramide C-8 GCs were measured using the spectrophotometric substrate DEVD-pNA, and representative bar graphs are shown for caspase-3 activity. Graphically, activities are represented as % of control group and all numerical values are represented as mean $\pm$ SEM of three individual experiments ($n = 3$). The groups with different alphabets are significantly different compared with respective parallel control ($P \leq 0.05$). **C.**

Membrane blebbing in adenovirus-directed knock-down PHB treated with or without ceramide C-8 (50 μ M) GCs. Cells were stained for phosphatidylserine with Alexa 488-conjugated annexin V and propidium iodide (PI) as a vital stain and viewed under epifluorescent illumination and by phase contrast microscopy. Images were captured at x400 magnification. Representative images are demonstrating the effect of ceramide C-8 at 8 h on GCs with knock-down PHB. Images a, c, e, and g are demonstrating staining specificity for annexin V. Images b, d, f and h are demonstrating phase picture of images a, c, e, and g respectively. The cells in early stages of apoptosis based on positive staining for annexin V but no uptake of PI at 8 h (images are not shown). Bar = 20 μ m. **D. Western blot analysis**

of proteins in adenovirus-directed knock-down PHB treated with or without ceramide C-8 (50 μ M) GCs. Western blot analysis of knock-down PHB expression in GCs treated with or without ceramide C-8 on cytochrome c released and caspase 3 activity with PHB knock-down. Equal amounts of protein (25 μ g) were applied to each lane and analyzed for knock-down Phb, cytochrome c released from mitochondria and caspase-3 activity. Representative blots were scanned using the NIH Image software program computer-assisted analysis system for quantitative assessment of changes in protein levels. Porin and α -tubulin were used as an internal control for mitochondria and cytosol respectively. The representative blots are used from three individual experiments (n = 3) performed to show the differences in expression pattern in proteins. AdshPHB – sh, Ad-scrambled – s.

Fig.4. Effects of recombinant adenovirus-directed overexpression of prohibitin (PHB) on ceramide C-8 induced apoptosis in granulosa cells (GCs)
 Overexpression of PHB (30-kDa) in GCs by transient infection with recombinant adenovirus constructs containing prohibitin sense cDNA (Ad-PHB). GCs were infected with or without the adenoviral PHB constructs for 2 h and cultured for 24 h in serum free media. After 24 h, media was replaced with fresh media with or without ceramide C-8 (50 μ M) in serum free media. Apoptosis was determined at different time (0, 2, 4, 8, 12, 16 and 24 h) interval. **A. Percent apoptosis in adenovirus-directed overexpressed prohibitin (PHB) treated with or without ceramide C-8 (50 μ M) GCs.** After each treatment cells were fixed and stained with Hoechst 33248 to identify nuclei. Data represent the percentage of cells displaying morphological alteration of apoptosis based on quantification of nuclear morphologic changes. At least 250-300 cells were counted for each data point. The bar graph represents the mean $\pm$ SEM of results from three replicate experiments. Significant differences are represented with different alphabets (a, b) at $P \leq 0.05$ in the respective panels. Same alphabets are showing insignificant differences. **B. Caspase-3 activity in adenovirus-directed overexpressed prohibitin (PHB) treated with or without ceramide C-8 (50 μ M) GCs.** Caspase-3 activity in cytosolic protein extracts from adenovirus directed with or without PHB treated with or without ceramide C-8 GCs were measured using the spectrophotometric substrate DEVD-pNA, and representative bar graphs are shown for caspase-3 activity. Graphically, activities are represented as % of control group and all numerical values are represented as mean $\pm$ SEM of three individual experiments ($n = 3$). The groups with different alphabets are significantly different compared with respective parallel control ($P \leq 0.05$). **C. Membrane blebbing in adenovirus-directed overexpressed prohibitin (PHB) treated with or without ceramide C-8 (50 μ M) GCs.** Cells were stained for phosphatidylserine with Alexa 488-conjugated annexin V and propidium iodide (PI) as a vital stain and viewed under epifluorescent illumination and by phase contrast microscopy. Images were captured at x400 magnification. Representative images are demonstrating the effect of over-expressed prohibitin on ceramide C-8 granulosa cells at 12h. Inset image of **a**

and **b** at a higher magnification, demonstrating staining specificity for annexin V. The cells in early stages of apoptosis based on positive staining for annexin V but no uptake of PI at 12 h (image not shown). **D. Western blot analysis of proteins in adenovirus-directed overexpressed prohibitin (PHB) treated with or without ceramide C-8 (50 μ M) GCs.** Western blot analysis of over-expressed PHB protein in GCs treated with or without ceramide C-8 on cytochrome c released and caspase 3 activity with PHB expression. Equal amounts of protein (25 μ g) were applied to each lane and analyzed for overexpressed PHB, cytochrome c released from mitochondria and caspase-3 activity. Representative blots were scanned using the NIH Image software program computer-assisted analysis system for quantitative assessment of changes in protein levels. Porin and α -tubulin were used as an internal control for mitochondria and cytosol respectively. The representative blots are used from three individual experiments ($n = 3$) performed to show the differences in expression pattern in proteins. **E.** The bar graph represents expression of PHB in the mean $\pm$ SEM from three replicate experiments after normalization of data against porin protein. Different alphabets indicate a significant difference ($P < 0.05$) when compared with control.

Fig.5.

A schematic model showing the adenovirus directed over-expression of prohibitin (PHB) in granulosa cells (GCs) leading to inhibition of apoptosis by inhibiting mitochondrial cytochrome c release to cytosol followed by inhibition of cytosolic caspase-3 activation.