Sphingolipid synthesis and role in uterine epithelia proliferation

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Abstract

Sphingolipids are involved in the regulation of cell proliferation. It has been reported that diacylglycerol and sphingosine-1-phosphate generation, during the synthesis of phospho-sphingolipids, is necessary for both, G1-S transition of cell cycle during the sustained activation of protein kinase C in various cell models (MDCK, *Saccharomyces* and *Entamoeba*) and AKT pathway activation. During the estrous cycle of the rat, AKT signaling is the main pathway involved in the regulation of uterine cell proliferation. The aim of the present study was to investigate the role of sphingolipid synthesis during proliferation of uterine cells in the estrous cycle of the rat. On metestrus day, when both luminal and glandular uterine epithelia present the maximal BrdU-labeled cells (S phase cells), there was an increase in the relative abundance of total sphingomyelins, as compared to estrus day. Myriocin, a sphingolipid synthesis inhibitor administered on estrus day, before the new cell cycle of epithelial cells is initiated, decreased the abundance of sphingomyelin, accompanied by proliferation arrest in uterine epithelial cells on metestrus day. In order to study the sphingolipid signaling pathway affected by myriocin, we evaluated the activation of the PKC-AKT-GSK3b-Cyclin D3 pathway. We observed that total and phosphorylated protein kinase C diminished in uterine epithelial cells of myriocin treated animals. Interestingly, cyclin D3 nuclear localization was blocked by myriocin, concomitantly with a decrease in nuclear pRb expression. In conclusion, we demonstrate that sphingolipid synthesis and signaling are involved in uterine epithelial cell proliferation during the estrous cycle of the rat.

Reproduction (2018) **156** 173–183

Introduction

Cellular proliferation and cell death in the uterus of rodents have been extensively studied and well characterized (Baranda-Avila *et al.* 2009). Many studies have been performed to determine estrogen and progestagen effects in these processes in ovariectomized rats and mice (Winuthayanon *et al.* 2014, Yuan *et al.* 2014). However, few studies have been performed to analyze estrous cycle regulation under physiological conditions (Mendoza-Rodriguez *et al.* 2003, Baranda-Avila *et al.* 2009).

The characterization of uterine epithelial cell proliferation and death patterns during the estrous cycle has been reported (Baranda-Avila *et al.* 2009, Mendoza-Rodríguez *et al.* 2009). Proliferation of epithelial cells in the rat uterus during the estrous cycle starts during the first hours of metestrus day (G1 to S) and finishes through proestrus day, involving the participation of the GSK3b/AKT pathway and the relocation of cyclins D1 and D3 from cytoplasm to the nucleus before starting epithelial proliferation (Furukawa *et al.* 1994, Stambolic

& Woodgett 1994, Huet *et al.* 1996, Altucci *et al.* 1997, Diehl *et al.* 1998, Alt *et al.* 2000, Grimes & Jope 2001, Doble & Woodgett 2003, Naderi *et al.* 2004, Chen *et al.* 2005, Mullany *et al.* 2008, Baranda-Avila *et al.* 2009, 2013, Kazi *et al.* 2009). Cyclin concentrations in the cell fluctuate during progression through G1, in response to mitogenic stimuli (Hunter & Pines 1994, Sherr 1994, Altucci *et al.* 1997, Mullany *et al.* 2008). Cyclins act predominantly by associating with the catalytic subunits of their specific cyclin-dependent kinase (CDKs) partners, cdk4 and cdk6. Their primary action is to phosphorylate members of the retinoblastoma (RB, p107 and p130) family, which results in release of E2F transcription factors (E2F 1–7) from an inhibitory RB complex and activation of genes required for cell cycle progression (Dalton 1992, Furukawa *et al.* 1994, Ohtani *et al.* 1995, Schulze *et al.* 1995, Tommasi & Pfeifer 1995, Botz *et al.* 1996, Geng *et al.* 1996, Huet *et al.* 1996). It is well known that estradiol is the best mitotic inducer of cellular proliferation studied in this tissue. However, it has been demonstrated that under physiological

conditions, during the estrous cycle, ICI 182,780, one of the most complete and the best characterized estradiol receptor (ER) antagonists in both *in vitro* and *in vivo* models, was only able to moderately inhibit luminal but not glandular uterine epithelial proliferation when administered at 09:00h of estrus day. Therefore, during the estrous cycle, anti-estrogens do not efficiently inhibit cell proliferation, as they do in ovariectomized (ovx) animals (Baranda-Avila *et al.* 2013), suggesting the participation of other molecules in the regulation of uterine epithelial cell proliferation under physiological conditions (Winuthayanon *et al.* 2014).

Sphingolipids are essential components of cellular membranes that are involved in a great number of pivotal cell functions. Sphingolipid synthesis *de novo*, initiates with the condensation of serine and palmitoyl CoA via serine palmitoyltransferase (SPT) to form 3-ketodihydrosphingosine (Menaldino *et al.* 2003). It is well known that sphingolipids participate in many cellular functions, such as cell cycle progression, cell death, differentiation, senescence, autophagy and migration (Marsh *et al.* 1995, Perry 2002, Hannun & Obeid 2008, Young *et al.* 2013). However, their participation in cell proliferation in animal models under physiological conditions has not been explored.

Previous studies on the role of sphingolipids in the cell cycle have been based on the use of myriocin, which inhibits SPT ($K_i=0.28\text{ nM}$), an enzyme that catalyzes the first and rate-limiting step of sphingolipid biosynthesis causing depletion of in membranes (Miyake *et al.* 1995). Myriocin also decreases extracellular sphingomyelin (SM), sphingosine-1-phosphate (S1P) and glycosphingolipid levels (Hojjati *et al.* 2005, Glaros *et al.* 2007). The molecular mechanisms responsible for the inhibition of SPT by myriocin have been described (Chen *et al.* 1999). There are reports that sphingolipids mediate cell proliferation via activation of the AKT pathway (Baudhuin *et al.* 2002, Kim *et al.* 2003, Balthasar *et al.* 2006, Gangoiti *et al.* 2008, Li *et al.* 2017). Furthermore, it has been found that the diacylglycerol (DAG) generated during the synthesis of SM, in MDCK cells, is required for the sustained activation of protein kinase C (PKC) during G1 to S phase transition and DNA synthesis (Cerbón & del Carmen Lopez-Sanchez 2003, Cerbón *et al.* 2005, 2009). It has also been reported that myriocin inhibits the proliferation of superantigen-stimulated peripheral blood mononuclear cells, without inducing cell death (Blank *et al.* 2005). In melanoma cells, inhibition of sphingolipid synthesis by myriocin results in G2/M arrest of the cell cycle and growth inhibition (Lee *et al.* 2011). Given the huge cellular proliferative processes occurring during the estrous cycle, we speculate that sphingolipid synthesis and signaling could be associated with uterine epithelial cell proliferation, which leads to cyclic epithelial growth and turnover.

The aim of our present study was to investigate the role of sphingolipids on AKT signaling during the G1-S transition in uterine epithelial cells during the estrus to metestrus transition of the estrous cycle of the rat, under physiological conditions.

Materials and methods

Animals

All animals and procedures were conducted according to the Official Mexican Norm (NOM-062-ZOO-1999) in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the USA and with the approval of the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL), from Facultad de Química, UNAM. For this study, we used intact adult female Wistar rats (200–250 g), which had at least four regular 4-day estrous cycles, determined by daily vaginal smears. The animals were kept under 12:12-h light:darkness cycles, lights on from 06:00 to 18:00h, with food and water available *ad libitum*.

Taking advantage that we and others had previously described the proliferation pattern of epithelial cells in the endometrium during estrous cycle in detail (Baranda-Avila *et al.* 2013), we designed a series of experimental treatments adding myriocin, just before proliferation initiation (throughout estrus day), for sphingolipid synthesis inhibition, as previously described (Lee *et al.* 2012). The tested dosage of myriocin was determined based on previous studies in rodents (Johnson *et al.* 2004, Osuchowski *et al.* 2004, He *et al.* 2005, Lee *et al.* 2012). Four groups of four animals each were formed. The first group was i.p. injected with myriocin (Sigma) in sterile phosphate buffer saline (PBS, pH 7.4), at a dose of 1 mg/kg twice, at 09:00 and 17:00h, on estrus day (subsequently called myriocin 09:00E). The animals of the second group were administered the same dose of myriocin, at 17:00 and 24:00h of estrus day (subsequently called myriocin 17:00E) (Baranda-Avila *et al.* 2009). The third and fourth groups were treated with vehicle (sterile PBS, pH 7.4) at the same times (control groups). All animals were killed at 13:00h on metestrus so that all the samples were in the same stage of the estrous cycle. One uterine horn of each animal was immediately fixed with ice-cold 4% paraformaldehyde in PBS for 2 h. After fixation, the tissues were dehydrated through a series of solutions with increasing ethanol concentrations, cleared with xylene and were then embedded in paraffin using routine histological procedures.

In order to study the differences in uterine epithelium lipid composition between a proliferating state (metestrus day) and a non-proliferating state (estrus day), we used another group of 4-day estrous cycle that received no treatment. Briefly, for lipid extraction experiments from uterine epithelial cells, uterine horn was longitudinally cut and the epithelium was removed from the endometrium by a gentle scraping with a needle bevel 25G caliber. The epithelial cells were washed with PBS and collected by centrifugation at 800g at 4°C for 5 min. PBS was discarded and the cells were suspended in

methanol. Samples were protected from light and were stored at 4°C until lipid extraction was performed.

Lipid extraction

Lipids were extracted using the methanol stop procedure, as previously reported (Van Veldhoven & Bell 1988, Cerbón *et al.* 2009). In brief, uterine epithelial cells suspended in methanol were heated at 54°C during 30 min. After cooling, the tubes at room temperature, chloroform/methanol (2/1) was added. Preparations were centrifuged at 4400g for 10 min, the supernatant was recovered and treated with alkaline solution (0.1 M KOH in methanol) for 2 h in an ice bath and neutralized with glacial acetic acid to remove glycerophospholipids. Sphingolipids were extracted with chloroform/methanol (2/1). The lower, organic phase was separated and dried under nitrogen and dissolved in a known volume of methanol/chloroform (2/1) and formic acid 0.1% for mass spectrometry analysis in positive mode, using the ESI ionization 3200 Q-TRAP LC/MS/MS AB SYSTEM. A scan of precursors for *m/z* 184.4 was used to detect the subspecies of SM, and scans of the precursors for *m/z* 264.4 (d18:1 backbone) and 266.4 (d18:0 backbone) were performed over a wide range of collision energies (30–60 eV). The results were analyzed following the methods previously described (Merrill *et al.* 2005).

BrdU immunostaining

Two hours before uterine collection, each animal received an i.p. injection of 5-bromo-2'-deoxyuridine (BrdU) in PBS at a dose of 50 mg/kg. All animals were euthanized at 13:00 h on the day of metestrus. Uterine tissue sections (5 µm thick) were cut and mounted on poly-L-lysine (Sigma) coated slides. The BrdU Labeling and Detection Kit (Roche) was used to detect cells that incorporated BrdU into their DNA, following the manufacturer's protocol. First, rehydrated sections were microwaved (two cycles of 10 min each) in 0.01 M sodium citrate, pH 6. Then, slides were washed with PBS and incubated in 0.5% Triton X-100 (Sigma) for 10 min, and non-specific immunoglobulin binding was blocked by incubating sections in 5% bovine serum albumin (BSA) (Research organics, Cleveland, OH, USA) for 30 min. Sections were incubated with an anti-BrdU mouse monoclonal antibody at a 1:10 dilution in incubation buffer (66 mM Tris buffer, 0.66 mM MgCl₂, 1 mM mercaptoethanol), for 1 h at 37°C. As negative control, primary antibody was omitted. After washing with PBS, sections were incubated for 2 h at room temperature with fluorescein-conjugated rabbit anti-mouse IgG diluted 1:10 in PBS. Slides were washed with PBS and covered with DAKO Fluorescent Mounting Medium (DAKO Corporation). Sections were observed in an Eclipse E600 fluorescent microscope (Nikon Co., Ltd.), using a B-2 filter from Nikon.

DNA synthesis in the luminal and glandular epithelia (LE and GE respectively) was assessed by counting the number of BrdU-positive nuclei in a section and expressed as percent of the total number of epithelial cells. Five sections per animal were analyzed. *N*=4 animals per experiment, and all experiments were performed in triplicate. 40× magnification images were used for each of the animals.

Immunohistochemistry

Tissue sections (5 µm thick) were cut and mounted on poly-L-lysine-coated slides. Sections were cleared and deparaffinized with xylene, rehydrated through a series of decreasing ethanol concentrations and processed for immunohistochemistry. Briefly, rehydrated sections were microwaved (two cycles of 10 min each), in 0.01 M sodium citrate, pH 6 and cooled at room temperature. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in PBS for 30 min. After rinsing in PBS, slides were incubated in 0.5% Triton X-100, and non-specific immunoglobulin binding was blocked by incubating sections in 5% BSA for 1 h. Sections were incubated with primary antibody at the specified dilutions: PKC: 1:200; AKT: 1:500; p-AKT 1/2/3 (Ser 473): 1:500; GSK3β: 1:500; p-GSK3β (Ser 9): 1:500; Rb: 1:200; cyclin D3: 1:100 (sc-80, sc-8312, sc-7985-R, sc-9166, sc-11757-R, sc-7905, sc-182, respectively, Santa Cruz Biotechnology, Inc.), pPKCα/βII (Thr 638/641): 1:200; pRb (Ser807/811): 1:200 (#9375, #8516, respectively, Cell Signaling Technology, Inc) in a humidified chamber at 4°C overnight. As negative control, primary antibody was omitted. After washing with PBS, sections were incubated for 2 h at room temperature with biotin-conjugated anti-rabbit IgG (Vector, Vectastain ABC KIT, Burlingame, CA, USA) at a 1:200 dilution and then washed with PBS to remove unbound secondary antibody and incubated for 1 h in peroxidase-conjugated avidin-biotin reagent (Vector, Vectastain ABC KIT). The slides were washed with PBS, developed with DAB substrate reagent (Vector, Peroxidase Substrate Kit) and counterstained with Mayer's hematoxylin. The slides were analyzed under a Nikon E600 microscope coupled to a DS-5M Nikon digital camera. Nuclei and cytoplasmic staining were analyzed using Image J software (NIH). For determining staining intensity (mean gray value) of immunopositive cells, RGB (red-green-blue) images were deconvolved to calculate the contribution of each of the applied stains (DAB and hematoxylin), based on the stain-specific RGB absorption (Ruifrok & Johnston 2001, Ruifrok *et al.* 2003). DAB images were converted to grayscale images. The intensity of staining (mean gray value), of the immunopositive cells was evaluated using Image J software. The gray level was converted into a numerical value on a scale from 0 (white) to 255 (black). The background was subtracted from all values. A total of 100 cells of each cell type per section were analyzed. Three sections per animal were analyzed. *N*=4 animals per group.

Statistical analysis

Total SM, BrdU and immunohistochemistry data were analyzed using one-way ANOVA followed by a Tukey's multiple comparison test. The Prism 6.01 program (GraphPad) was used for calculating probability values.

Results

Sphingolipid profile in uterine epithelial cells

Sphingolipids are involved in uterine epithelial cell proliferation *in vivo*. In order to determine sphingolipid synthesis *in vivo* and its participation in uterine epithelial

cell proliferation from isolated uterine epithelial cells (as described in 'Materials and methods' section), we first determined the species of SM on tissue samples from estrus and metestrus days with or without treatment with myriocin. As can be seen in Fig. 1A, SM subspecies were detected in both estrus and metestrus periods. In the uterine epithelia on metestrus day, sphingolipid content was more abundant as compared with estrus day. In the presence of myriocin, there was a significant decrease in the abundance of SM species (Fig. 1A and B). The most abundant species detected were SM (34:1), (36:1), (37:0), (38:1), (42:3) and (42:1). As shown in Fig. 1B, the content detected of these sphingolipids during metestrus was markedly higher compared to those during estrus (Fig. 1 and Table 1).

Figure 1 Sphingomyelin levels in the rat uterus increase during the metestrus day of the estrous cycle and are reduced by myriocin. (A) Total sphingomyelins in the rat uterus during the estrus and metestrus days of the estrous cycle of animals previously treated with myriocin, as indicated in materials and methods. Results are expressed as the mean $\pm$ S.E.M. (B) All main individual molecular species of sphingomyelins were identified using mass spectrometry, as described in materials and methods. * $P < 0.05$ estrus vs metestrus. ** $P < 0.05$ metestrus vs metestrus + myriocin. $n = 4$ per group.

Table 1 Main sphingomyelin subspecies in the rat endometrium during estrous and metestrous days of the estrous cycle.

Sphingomyelin subspecies	Vehicle	Myriocin
703.8 (34:1)	2.1	0.9
731.8 (36:1)	3.5	0.75
746.9 (37:0)	2.57	1
759.8 (38:1)	2.67	0
811.9 (42:3)	1.6	0
815.9 (42:1)	2.5	0

Sphingomyelin subspecies values of mass spectra, in parenthesis long-chain base.

Sphingolipid synthesis inhibition by myriocin affects uterine cell proliferation

The effects of sphingolipid synthesis inhibition by myriocin, on the G1-to-S phase transition of uterine epithelial cells, were assessed by BrdU incorporation. In the myriocin 09:00E group, there was a significant reduction of BrdU-labeled cells in both LE and GE (Fig. 2B and E), as compared to the control group (Fig. 2A and D). In contrast, in the myriocin 17:00E group, the inhibitor only significantly diminished proliferation in LE (Fig. 2C and F), as compared with vehicle-treated animals (Fig. 2A and D). This observation is in line with the differential timing of LE and GE proliferation initiation, GE starts at least 3 h before LE, as previously described (Baranda-Avila *et al.* 2009).

Myriocin inhibits PKC, AKT and GSK3 phosphorylation

In order to assess the signaling pathways involved in myriocin inhibition of G1-S transition, we evaluated both total and phosphorylated forms of PKC, AKT and GSK, in uterine epithelial cells.

PKC expression was mainly localized in the cytoplasm of both LE and GE in vehicle-treated animals, with few immunopositive nuclei (Fig. 3A). A significant reduction in PKC immunostaining was observed in both epithelia in the myriocin 09:00E group (Fig. 3B and J) as compared to the vehicle-treated group (Fig. 3A). In contrast, in the myriocin 17:00E animal group, no significant difference in PKC immunostaining was observed in both uterine (luminal and glandular) epithelia, as compared to the vehicle group (Fig. 3C and J).

We also evaluated the expression of phosphorylated pPKC α and β (activated by DAG) in the different treatment groups (Fig. 3D, E and F). In contrast to PKC, pPKC was mainly localized in the nuclei of uterine epithelial cells in vehicle-treated animals, with some cytoplasmic staining in LE (Fig. 3D). A significant decrease in pPKC nuclear staining in both uterine epithelia was observed in animals treated with myriocin at 09:00E, as compared to vehicle-treated animals (Fig. 3E and K). In addition, no cytoplasmic staining was observed in both epithelia. Uterine glandular epithelial cells from the animals in the myriocin 17:00E group presented similar staining

Figure 2 Myriocin treatment inhibits G1-S transition of the cell cycle (reduced the BrdU-labeled nuclei) in rat epithelial cells. G1-S transition of the cell cycle was evaluated at 13:00h of metestrus day in uterine epithelial cells by BrdU incorporation. Animals were administered with vehicle (A and D) or myriocin at 09:00 and 17:00h of estrus day (B and E) or myriocin at 17:00 and 24:00h of estrus day (C and F). (G, H and I) Negative controls, where primary antibody was omitted. (J) Quantitative representation of BrdU incorporation in the uterine epithelia during metestrus day. Immunofluorescent nuclei were determined in luminal epithelium (black bars) and glandular epithelium (white bars). Results are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ as compared with vehicle-treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 100 μ m. $n = 4$ per group.

to the vehicle-treated animals (Fig. 3F vs D). However, luminal epithelial cells presented a slight non-significant increase in the number of immunopositive nuclei, and cytoplasmic staining was lost, as compared to vehicle-treated animals (Fig. 3F vs D).

In control animals, Akt immunostaining was detected in the nuclei and cytoplasm of uterine epithelia of vehicle-treated animals (Fig. 4A). The myriocin 09:00 E group presented a decrease in Akt immunostaining (Fig. 4B), as compared to the control group (Fig. 4A).

Figure 3 Myriocin administered at 09:00h of estrus inhibits PKC and PKC phosphorylation. PKC and pPKC were evaluated by immunohistochemistry in the uterine epithelia of animals treated with vehicle (A and D), myriocin at 09:00 and 17:00h of estrus day (B and E), or myriocin at 17:00 and 24:00h of estrus day (C and F). (G, H and I) Negative controls, where primary antibody was omitted. All animals were sacrificed at 13:00h of metestrus day. (J) PKC immunohistochemistry was analyzed quantitatively using Image J software, as described in materials and methods. (K) In pPKC immunohistochemistry, where nuclear staining was predominant, the percentage of positive nuclei was assessed as described in materials and methods. Results are expressed as the mean $\pm$ S.E.M. Black bars represent the luminal epithelium (LE) and the white bars the glandular epithelium (GE). * $P < 0.05$ as compared with the vehicle treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μ m. $n = 4$ per group.

This decrease was more evident in the cytoplasm of both uterine epithelia. In contrast, the myriocin 17:00 E group presented a significant increase in Akt expression in both uterine epithelia (Fig. 4C and J), as compared to control animals (Fig. 4A and J).

The vehicle-treated animals presented pAkt expression in the cytoplasm and nuclei of both uterine epithelia (Fig. 4D). Animals in the myriocin 09:00 E group presented a significant decrease in staining intensity in both epithelia, as compared to the control group (Fig. 4E and K vs D and K). Interestingly, in this group, the decrease in pAkt expression was observed both in the nuclei and in the cytoplasm (Fig. 4E). Additionally, the animals in the myriocin 17:00 E group, presented a significant increase in the staining intensity in the cytoplasm and nuclei of both uterine epithelia, as compared to the control group (Fig. 4F and K vs D and K).

Figure 4 Myriocin administered at 09:00 h of estrus inhibits Akt phosphorylation. Akt and pAkt were evaluated by immunohistochemistry in the uterine epithelia of animals treated with vehicle (A and D), myriocin at 09:00 and 17:00 h of estrus day (B and E), or myriocin at 17:00 and 24:00 h of estrus day (C and F). (G, H and I) Negative controls, where primary antibody was omitted. All animals were sacrificed at 13:00 h of metestrus day. (J and K) Immunohistochemistries were analyzed quantitatively using Image J software, as described in materials and methods. Results are expressed as the mean $\pm$ S.E.M. Black bars represent the luminal epithelium (LE) and the white bars the glandular epithelium (GE). * $P < 0.05$ as compared with vehicle treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μ m. $n = 4$ per group.

pAkt phosphorylates GSK3 β , inhibiting its function. GSK3 β phosphorylates cyclin D3 at Thr286, thereby targeting it for nuclear export and proteolysis. In vehicle-treated animals, we observed nuclear and cytoplasmic staining of GSK3 β (Fig. 5A). In both groups of myriocin-treated animals, a significant increase in GSK3 β expression in nuclei and cytoplasm of both epithelia was observed, as compared to control animals (Fig. 5B, C and J).

In vehicle-treated animals, pGSK3 β was localized in the nuclei and cytoplasm of both uterine epithelia (Fig. 5D). pGSK3 β only presented a small but significant decrease in LE in both myriocin-treated groups, as compared to control animals. GE of both myriocin groups of animals did not present significant differences

Figure 5 Myriocin induces GSK3 β . GSK3 β and pGSK3 β were evaluated by immunohistochemistry in the uterine epithelia of animals treated with vehicle (A and D), myriocin at 09:00 and 17:00 h of estrus day (B and E), or myriocin at 17:00 and 24:00 h of estrus day (C and F). (G, H and I) Negative controls, where primary antibody was omitted. All animals were sacrificed at 13:00 h of metestrus day. (J and K) Immunohistochemistries were analyzed quantitatively using Image J software, as described in materials and methods. Results are expressed as the mean $\pm$ S.E.M. Black bars represent the luminal epithelium (LE) and the white bars the glandular epithelium (GE). * $P < 0.05$ as compared with the vehicle treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μ m. $n = 4$ per group.

in pGSK3 β expression, as compared to control animals (Fig. 5E, F and K vs D and K).

We have previously reported (Baranda-Avila *et al.* 2013) AKT Ser 473 phosphorylation in the uterus of cycling animals in metestrus day by Western blot, but GSK3 β phosphorylation at Ser9 was minimal or below detection.

Myriocin inhibits cyclin D3 nuclear localization

Previous studies demonstrated that cyclins D1 and D3 are key molecules in uterine epithelial proliferation (Baranda-Avila *et al.* 2009). In fact, it has been demonstrated that cyclin D3 was dramatically changed by its phosphorylation and relocalization from cytoplasm to the nucleus during cell proliferation in this tissue (Baranda-Avila *et al.* 2009). To further assess the molecular mechanism involved in myriocin inhibition of proliferation, we investigated cyclin D3 nuclear localization. In control animals, cyclin D3 was localized

in the nuclei and cytoplasm of both LE and GE (Fig. 6A and D). In contrast, in both groups of myriocin-treated animals, 09:00E (Fig. 6B and E) and 17:00E (Fig. 6C and F), practically no nuclear localization of cyclin D3 was observed, while cytoplasmic expression of the protein was maintained (Fig. 6).

Myriocin prevents Rb phosphorylation

As shown in Fig. 7, in vehicle-treated animals Rb was localized in both the nuclei and cytoplasm of GE and LE (Fig. 7A). In the 09:00E myriocin group, cytoplasmic Rb protein expression was decreased in LE, while it presented a similar expression in GE, as compared to control animals (Fig. 7B and J). Meanwhile, similar Rb protein expression was observed in the myriocin 17:00E group (Fig. 7C and J), as compared to the control group.

In vehicle-treated animals, pRb was predominantly localized in the nuclei of GE and LE (Fig. 7D). Interestingly, in the 09:00-h myriocin group, a significant decrease in the number of pRb immunopositive nuclei was observed, in both epithelia (Fig. 7E and K). Similarly, the myriocin 17:00E group, also showed a significant decrease in the number of immunopositive nuclei in both epithelia (Fig. 7F and K).

Discussion

The present study describes, for the first time, sphingolipid synthesis pattern in uterine epithelial cells of the rat during the beginning of its proliferation in estrus–metestrus cycle progression (G1-to-S transition). We also found that sphingolipid synthesis is required for epithelial cell proliferation.

The rat uterus has been extensively used as a model for the study of molecular mechanisms of cellular proliferation (Yuan *et al.* 2014). It is well known that the uterine epithelium of rodents and humans present cycles of massive cell proliferation and differentiation under the regulation of 17 β -estradiol and progesterone (Winuthayanon *et al.* 2014, Yuan *et al.* 2014). However, during the estrous cycle, the use of specific estrogen receptor antagonists is only able to moderately inhibit cell proliferation when administered under physiological conditions, indicating that there are other molecules participating in this process (Baranda-Avila *et al.* 2013).

It is well known that the sphingolipid biosynthesis pathway generates bioactive molecules crucial to the regulation of cellular physiological processes. It has been reported that DAG generated during SM synthesis plays an important role in PKC activation, necessary for transit through the cell cycle and cellular proliferation in different cellular models (Blank *et al.* 2005, Cerbón *et al.* 2005). In the present study, we found that in rat uterine epithelial cells, there is an increase in SM synthesis during metestrus day (Fig. 1A), when proliferation begins and the highest BrdU labeling of epithelial cells in this

Figure 6 Myriocin inhibits cyclin D3 nuclear localization. Cyclin D3 was evaluated by immunohistochemistry in the uterine epithelia of animals treated with vehicle (A and D), myriocin at 09:00 and 17:00h of estrus day (B and E), or myriocin at 17:00 and 24:00h of estrus day (C and F). All animals were sacrificed at 13:00h of metestrus day. (A, B and C) Show luminal epithelium. (D, E and F) Show glandular epithelium. (G, H and I) Negative controls, where primary antibody was omitted. (J) Percentage of immunopositive nuclei were evaluated as described in materials and methods. Results are expressed as the mean $\pm$ S.E.M. Black bars represent the luminal epithelium (LE) and the white bars the glandular epithelium (GE). * $P < 0.05$ as compared with the vehicle treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μ m. $n = 4$ per group.

tissue is observed (Baranda-Avila *et al.* 2013). We also observed that the inhibition of SM synthesis prevents G1-to-S phase transition of the epithelial cells (Fig. 2J). This observation correlates well with the inhibition of SM synthesis *in vivo* obtained by administration of myriocin (Fig. 1), indicating that sphingolipid synthesis is required for estrous cycle cell proliferation (Baranda-Avila *et al.* 2013). However, further studies are need to establish whether changes in cell metabolism and membrane properties, as a consequence of sphingolipid depletion by myriocin, can induce apoptotic cell death leading to inhibition of cellular growth, in a similar manner as was previously reported in lung cancer cells

Figure 7 Myriocin prevents Rb phosphorylation. Rb, and pRb were evaluated by immunohistochemistry in the uterine epithelia of animals treated with vehicle (A and D), myriocin at 09:00 and 17:00 h of estrus day (B and E), or myriocin at 17:00 and 24:00 h of estrus day (C and F). (G, H and I) Negative controls, where primary antibody was omitted. All animals were sacrificed at 13:00 h of metestrus day. Immunohistochemistry of Rb, where nuclei and cytoplasmic staining was observed, was analyzed quantitatively using Image J software, as described in materials and methods (J). pRb immunohistochemistry, where nuclear staining was predominant, the percentage of positive nuclei was assessed as described in materials and methods (K). Results are expressed as the mean $\pm$ S.E.M. Black bars represent the luminal epithelium (LE) and the white bars the glandular epithelium (GE). * $P < 0.05$ as compared with the vehicle treated animals. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μ m. $n = 4$ per group.

(Choi *et al.* 2014). Moreover, the use of many sphingolipid-derived reagents has been described, and they are useful tools to further explore the molecular mechanisms involved in sphingolipid synthesis and homeostasis (Chen *et al.* 1999). We next investigated whether the inhibition of SM synthesis affects the molecular signaling pathway involved in uterine epithelial cell proliferation. Since myriocin inhibits the generation of DAG, which induces the phosphorylation and activation of PKC, we evaluated PKC and its phosphorylation at Thr638 (PKC α) or Thr641 (PKC β II). We observed a decrease in both PKC and pPKC in animals treated at 09:00 h of estrus day (Fig. 3B and E), as compared with the vehicle-treated animals (Fig. 3A and D). After SM synthesis inhibition by myriocin, we observed a concomitant inhibition of proliferation in both uterine LE and GE (Fig. 2), thus indicating that inhibition of PKC and its activation to pPKC modifies the proliferation pattern of these cells.

In contrast, in animals treated with myriocin at 17:00 E day, only the proliferation of LE was inhibited as compared to the vehicle-treated animals (Fig. 2J). pPKC nuclear localization has been previously observed, and this fact evidences the possibility that signal transduction events could also occur at the nuclear level during the induction of cell proliferation (Alessenko & Burlakova 2002). It has been reported that PKC, particularly PKC α and PKC β 1 isoforms, phosphorylate GSK-3 β *in vitro* (Goode *et al.* 1992, Pap & Cooper 1998, Fang *et al.* 2002), and this phosphorylation results in its specific inactivation.

It has been described that activation of Akt via pPKC occurs during epithelial cell proliferation (Merrill *et al.* 2005). In this study, we found that reduction of SM synthesis after myriocin administration modifies the normal activation of this pathway at different levels, inducing a significant decrease of pAkt proteins in both epithelia in the 09:00 myriocin group (Fig. 4K). This reduction was observed, both in the nuclei and cytoplasm of epithelial cells. This result is in line with previous reports indicating that when Akt is activated it detaches from the inner surface of the plasma membrane, where it is initially activated and relocates to the nucleus (Downward 1998, Datta *et al.* 1999). Therefore, the decrease in pAkt in the cytoplasm and nuclei of uterine epithelia indicates a decrease in Akt phosphorylation.

GSK3 β phosphorylates cyclin D3 at Thr283, thereby targeting it for nuclear export and proteosomal degradation (Naderi *et al.* 2004). In the vehicle-treated group, there was a predominant staining of pGSK3 β over GSK3 β (Fig. 5A and D, J and K). Conversely, in myriocin treated animals, GSK3 β staining predominated over pGSK3 β (Fig. 5B, C, E, F, J and K). This increase of GSK3 β observed in myriocin-treated animals correlates well with the decrease in cyclin D3 nuclear localization observed in both epithelia (Fig. 6B, C, E, F and J), indicating that cyclin D3 remains outside the nuclei and may be processed for its degradation.

It has been well established that Cyclin D3 plays a central role in the G1-to-S phase cell cycle transition, by phosphorylating Rb. Phosphorylation of Rb results in the release of E2F transcription factors from an inhibitory Rb complex and activation of genes required for cell cycle progression, such as cyclin E, cyclin A, cdc2 and cdk2 (Grimes & Jope 2001, Doble & Woodgett 2003, Naderi *et al.* 2004). As shown in Fig. 7, in the 09:00 E myriocin group, Rb expression was diminished in the LE, and there was a significant decrease of nuclear pRb in both epithelia (Fig. 7). These results indicated that myriocin treatment inhibits RB phosphorylation, and thus, further inhibition of cell cycle progression and proliferation (Fig. 2).

It has been recently demonstrated that dysregulation of sphingolipid metabolism occurs in different pathological conditions, such as endometriosis and different types of cancer, in which the proliferation process is altered (Aguilera-Romero *et al.* 2014, Lee *et al.* 2014).

In addition, it has been reported that the inhibition of sphingolipids by myriocin in glioma cells attenuates proliferation (Bernhart *et al.* 2015). Our results are in line with these observations, suggesting an important role for sphingolipids in cell cycle progression. Since sphingolipids are important mediators of both cellular proliferation and programmed cell death (Gangoiti *et al.* 2008, Choi *et al.* 2014, Li *et al.* 2017), it is important to investigate the precise role of individual sphingolipid species on uterine epithelial cell proliferation. Inhibitors downstream of SPT must be used in order to determine distinct roles of each species (Canals *et al.* 2011) and further elucidate the diverse pathways affecting uterine epithelial cell proliferation.

The overall results of this study indicate that *de novo* sphingolipid synthesis and signaling are key processes involved in uterine epithelial cell proliferation in the rat, under physiological conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by funding from The National Autonomous University of México PAIP and PAPIIT, IN216817 and grants from Consejo Nacional de Ciencia y Tecnología (CONACyT) 182189.

Acknowledgements

The authors thank I.B.T. Lorena Ramirez-Reyes from CINVESTAV-IPN (UGPM). Genomic, Proteomic and the Metabolomics Unit for SM analysis. They thank M.V.Z. Lucia Macias Rosales for her support in animal handling at the Faculty of Chemistry, UNAM vivarium.

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Received 30 October 2017

First decision 14 November 2017

Revised manuscript received 17 May 2018

Accepted 4 June 2018