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Subcellular Redistribution of NHERF1 in Response to Dehydroepiandrosterone (DHEA) Administration in Endometrial Glands of Wistar Rats

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ABSTRACT

To understand the regulation of Na(+)/H(+) exchanger regulatory factor (NHERF1) in polycystic ovarian syndrome (PCOS), we studied the expression of NHERF1 in uterus of Wistar rats injected with (6mg/kg) of dehydroepiandrosterone (DHEA) for 7 and 20 days. Immunohistochemistry analysis of NHERF1 showed a substantial shift in the intracellular localization of NHERF1 in endometrial glands and areas of luminal epithelium as early as 7 days of DHEA administration. NHERF1 accumulated in the "Golgi apparatus area" in virtually all of the glands in the 7-day protocol, and in the majority of the glands of the 20-day protocol. In contrast, NHERF1 is expressed in the apical membrane and slightly in the cytoplasm of the control epithelium. The subcellular redistribution of NHERF1 could affect the sorting of proteins to the apical membrane and the organization of the apical compartment.

KEYWORDS

NHERF1, Endometrium, Golgi Apparatus, PCOS

INTRODUCTION

Polycystic Ovarian Syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age and affects more than 5% of the population.[1] Though the etiology of PCOS is unclear, some evidence suggests that fetal exposure to high levels of androgens could predispose PCOS in adult life.[2] PCOS presents itself most commonly as a combination of anovulatory and ovulatory cycles, infertility and hyperandrogenicity and is associated with certain metabolic alterations like obesity, dyslipidemia, insulin resistance and type-2 diabetes, all of which increase the risk of cardiovascular disease.[3] It has been proven that both obesity and

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diabetes have a negative impact on fertility, indicating the importance of the prevention and treatment of clinical manifestations of PCOS.[4] The clinical manifestation of PCOS can be heterogeneous, and in cases of anovulatory cycles, the morphological changes most characteristic of PCOS are the accumulation of large antral follicles attributed to a failure in the selection of the dominant follicle and an arrest of development of follicles.[5,6] The hormonal changes and anovulatory cycles in women with PCOS can trigger hyperplasia of the uterine endometrium, which not only affects the process of implantation, but also increases endometrial cancer risk.[7] Even though the causes of PCOS are not completely understood, an increasing number of chemicals such as bisphenol A (BPA), diethylstilbestrol (DES), etc. are been studied in relation to their hormonal actions as endocrine disruptors and their effects in the reproductive system and the development of PCOS.[8] Of the several animal models of PCOS that have been developed such as rats treated with constant light, dehydroepiandrosterone (DHEA), testosterone propionate, estradiol valerate, letrozole, mifepristone (RU486), human chrorionic gonadotropin (Hcg) (hypothyroid rats) many showed some but not all of the alterations found in the human syndrome.[9, 10] The rat model of PCOS by administration of DHEA was first developed by Roy et al. in 1962 after the observation of an abnormal accumulation of this androgen in ovaries from women with PCOS.[11] This early report showed that the treatment with of 6 mg/100 g or higher doses of DHEA in immature rats produces changes in ovarian and uterine weight and follicular cyst formation. A cystic follicle is a large cyst filled with fluid with an attenuated granulose cell layer and thickened theca internal cell layer. [12, 13]

The function of the Na(+)/H(+) exchanger regulatory factor (NHERF1) protein in uterine physiology and its regulation has only recently begun to be studied, and its role in the alterations of the uterine epithelium found in pathological conditions like PCOS, insulin resistance and

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endometrial cancer remains unknown. NHERF1 protein is an adaptor molecule expressed primarily in the plasma membrane of polarized epithelia where signals connect the membrane with the cytoskeleton. NHERF1 participates in the organization of microvilli of polarized epitheliums; it regulates the activity of ion channels, growth factor receptors and the endocytic machinery.[14-16] In MCF-7 breast cancer cells, NHERF1 expression is regulated at the transcriptional level by estrogens, but not by other sexual steroids such as 5-dihydrotestosterone, testosterone or the progestin R5020.[17] Estrogens regulate gene expression by direct binding of the estrogen receptor to DNA palindromic consensus sequences of 13 base pairs called estrogen receptor elements or EREs. The consequent recruitment of transcriptional co-regulators determines the nature of the effect that could activate or inhibit gene transcription. NHERF1 expression is regulated by estrogens in human endometrium, where it is highly expressed in the proliferative phase of the menstrual cycle. [18] It was also characterized during the estrous cycle of Wistar rats, where the expression of NHERF1 was detected in all stages of the estral cycle in the apical and cytoplasm compartment, luminal membrane of glandular epithelium and scattered stromal cells. [19]

In polarized epitheliums, such as the endometrial-gland and luminal uterine epithelial cells, the asymmetrical distribution of lipids and proteins is maintained by the constant activity of the secretory and endocytic pathway.[20] The Golgi complex is the site of modification, classification and transport of glycoprotein and glycolipids to their final destination within the cells such as organelles or specific cell surfaces like the apical or basolateral membrane. It is also the place of synthesis for the majority of polysaccharides, such as glycosaminoglycans, which constitute the extracellular matrix of animal cells. It had been widely accepted that, in the trans-golgi network

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(TGN), proteins are classified according to their specific sorting signals, and they are targeted to different cell destinations.[21] However, recent reports support the notion that the sorting capacity is not exclusively of the TGN, rather it can occur in every step of the secretory pathway, introducing a new level of complexity to the currently puzzle of apical sorting signals.[22] A potential function of NHERF1 in the secretory pathway has started to be revealed. It was proposed that NHERF1 could influence the β-catenin/E-cadherin complex assembly and trafficking from the endoplasmic reticulum (ER) to the plasma membrane.[23] In MDCK renal epithelial cells, NHERF1 localization at the Golgi apparatus was required for the binding, oligomerization and sorting of the Podocalyxin/Gp135 protein to the apical membrane.[24] The association of NHERF1 to the Golgi apparatus opens a new paradigm, because NHERF1 is an adaptor protein that was traditionally associated with the submembrane cortical region.

The knowledge of the regulation of NHERF1 in uterus by hormones and endocrine disruptors could have very important implications in the basic understanding of uterine epithelial physiology, and have a direct impact in problems with fertility and cancer associated with metabolic disorders like PCOS. NHERF1 is an estrogen-regulated protein that has been implicated in uterine physiology and implantation, but the alteration of its expression in a complex hormonal context such as PCOS has never been explored. We believe that our results will help to understand how downstream action of estrogenic signals could be affected in this syndrome. Therefore, here we have studied the expression of NHERF1 in a rat model of PCOS induced by DHEA in order to contribute to the basic understanding of the cellular alterations that underlie the pathology of PCOS.

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MATERIALS AND METHODS

Animals and treatments

A group of 31 immature Wistar rats (22 day-old) weighing 35-40 grams was housed with a controlled temperature and light/dark cycle (lights on 6:00 AM to 8:00 PM) we included in the study. Rat chow (Gilardoni, Buenos Aires, Argentina) and tap water were available ad libitum. For the induction of the PCOS condition, a group of rats was injected daily with 6mg/100g b.w. of dehydroepiandrosterone (DHEA) (Sigma) diluted in sesame oil, and control rats were injected with vehicle. We performed two experimental protocols: one group of rats was injected with DHEA for 7 days, and another group was injected for 20 days. Rats in the 7-day protocol are considered immature (29 days old at the end of the treatment) where rats in the 20-day protocol had started the estrous cycle (43 days old at the end of the protocol). After treatment, the rats were anesthetized under a mixture of ketamine-xylazine anesthesia and euthanized. Samples of liver, kidney, uterus, and ovaries were collected. The whole ovary and uterus from the 20-day protocol rats were weighed. The ovary and medial sections of the uterus' horns were fixed immediately in PBS-buffered 10% formalin for 24 hours and processed for histological analysis. Animal maintenance and handling was performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication N8 86-23, revised 1985 and 1991), the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986) and the ethics guidelines suggested by the Argentinean Association of Science and Laboratory Animals (AACTyTAL).

Histological analysis

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After fixation, tissues were dehydrated and embedded in Histoplast Biopack (Buenos Aires, Argentina). Tissue sections (5 μm) were placed in 3-aminopropyltrietoxysilane (Sigma-Aldrich, St. Louis, MO, USA) coated slides and stained with hematoxylin-eosin for histopathological studies. Ovarian sections from control, 7-day and 20-day protocol were analyzed for the presence of follicles in different stages of folliculogenesis such as secondary (pre-antral), tertiary (antral), preovulatory (graafian) follicles, cystic follicles and corpus luteum. H&E slides from uterus were analyzed by a pathologist to describe epithelial morphology in control and DHEA injected rats. The diameter of the uterus was measured by analyzing the digital pictures of transversal uterine sections captured with a x4 objective (x40 total magnification) with an Olympus BX51 microscope coupled with an Olympus DP70 camera. Images were analyzed with the Image J program, and the diameter of the uterus and the height of the uterine epithelium (at x400 magnification) was calculated by establishing the correlation between pixels and μm using the calibrated scale bar at each magnification. Only uterine sections in the right transversal orientation were considered for quantification. The height of the uterine epithelium was measured in μm (10-20 fields/uterine section) from control and treated rats. The diameter of the largest follicle was measured as described above in control and treated rats from the 20-day protocol.

Immunohistochemistry and Immunofluorescence

Rabbit anti-NHERF1 polyclonal antibody was obtained from Affinity BioReagents (Rockford, IL, USA), mouse anti-TGN38 antibody from BD Transduction Laboratories (San Jose, California, USA). Unstained sections were used for immunohistochemistry analysis using two kits. The Vectastain Universal Elite ABC KIT, previous blocking of the samples with

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Avidin/Biotin blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) and the polymer based detection system NovoLink™ Min Polymer Detection System (cat. RE7290-K, Bioars, Buenos Aires, Argentina) were used according to manufacturers instructions. The experiment was repeated three times. We counted the amount of total glands per uterine section, and we classified the NHERF1 label in each gland as: apical/citoplasmic or supranuclear/golgi area staining. The total number of fields analyzed corresponds to the quantification of glands in 2 consecutive sections/rat from the 7-day protocol and 4 consecutive sections from the 20-day protocol from 4-7 rats per group. The immunofluorescence protocol was performed in sections of paraffin embedded tissues. Briefly, the tissue slides were deparaffinized by heating them in an oven at 60 degrees for 30 minutes, cooled down and placed three times in xylene for 5 minutes. The slides were then hydrated twice in 100% ethanol, once in 95% and 70% ethanol and transferred to ddH20. For antigen retrieval, the slides were incubated for 20 min in boiling citrate buffer in a steamer, cooled for 10 min, rinsed 3 times (5 minutes each) with ddH20 and transferred to PBS. Unspecific binding was avoided by incubation with blocking solution for 20 minutes (Vector Laboratories, Inc., Burlingame, CA, USA) followed by incubation with primary antibody diluted in PBS in a humidified chamber overnight. The slides were then washed 3 times with PBS for 5 min, incubated with a FITC anti-mouse IgG secondary antibody for 30 min, rinsed 3 times with PBS for 5 min and mounted with Vectashield -DAPI aqueous solution medium (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistical Analysis

The differences between control and DHEA injected groups were compared using the GraphPad Prism 5 program (GraphPad Software, Inc. La Jolla, CA, USA) using an unpaired *t* test.

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RESULTS

Morphological and histological changes in uterus and ovaries after DHEA administration

Here we induced a PCOS condition in 22 day-old Wistar rats that were treated for 7 and 20 days by a daily subcutaneous administration of DHEA (6mg/kg), and we observed substantial changes in tissue and cell architecture as early as 7 days of DHEA injection. Taking into consideration that most of the studies of PCOS induction have been characterized in Sprague Dawley rats and mice, we analyzed the morphological and histological changes observed in our experimental conditions. [25, 26] Consistent with previous reports, the administration of DHEA to 22 day-old rats had a significant effect in the morphology and histology of the uterus. Rats treated with DHEA for 7 days displayed ovaries that were larger than controls (Fig. 1A-B) with accumulation of tertiary follicles; some of them presenting oocyte fragmentation and atresia, as previously described.[26] In the ovaries of rats treated for 20 days with DHEA, several follicular cysts occupied most of the ovary, and they showed a decreased granulose cell wall and cellular debris in the cyst cavity. All of the control ovaries contained corpora lutea consistent with a normal reproductive cycle (Fig. 1C-D). Uteri of DHEA-treated rats for 7 days were enlarged and underwent morphological changes in the uterine epithelial cells. Uterus of control animals showed a conserved cuboidal epithelium (Fig. 1E-F). The uterus of the 20-day DHEA-treated group showed an alteration of the epithelial layer with cell vacuolization, nuclear hipercromatism and areas of hyperplasia. Two rats presented a more severe phenotype with areas of epithelial dysplasia (Fig. 1G-H). In order to quantify the changes observed in the uterus, pictures of tissue sections were captured and analyzed with Image J software. Uteri from 7-day DHEA treatment

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showed a significant increase in the uterine diameter (mean ± SD) 1.52 ± 0.15 mm compared to controls 1.15 ± 0.14 mm (p=0.0041) and the height of the epithelial cells 29.68 ± 9.87 μm compared to control uterus 14.90 ± 1.08 μm (P< 0.05) (Fig. 2A and B). In rats treated for 20 days with DHEA, we observed a significant change in ovarian and uterine weights. Ovaries from treated rats were smaller 19.85 ± 4.44 mg vs. controls 36 ± 7.35 mg (p=0.0094), whereas the uteri were heavier than controls, 276.7 ± 36.1 mg vs. 196.5 ± 51.0 mg (p<0.05) (Fig. 2C and D). The diameter of the largest follicle in 20-day treated rats was significantly larger 649.3 ± 57.9 μm compared to the control rats 522.2 ± 48.2 μm (p<0.05).

NHERF1 expression in uteri of normal and DHEA-treated rats

In the uterine epithelia, we found a substantial change in NHERF1 subcellular localization after the induction of an experimental PCOS condition in immature Wistar rats. The immunohistochemistry analyses were confirmed using two detection systems as it was described in materials and methods. Samples of kidney and liver, from vehicle injected animals, were included as control tissues where NHERF1 expression has been previously well characterized (Fig. 3. A-B).[15, 27] We analyzed uterine tissue sections from control and treated rats, and we found that, in the controls, NHERF1 is expressed in the apical membrane and slightly in the cytoplasm of the glandular and luminal epithelial cells in both protocols. Our more striking and consistent finding in both the 7-day and 20-day protocol was the substantial shift in the intracellular localization of NHERF1 to the supranuclear region or "Golgi apparatus area" in the uterine glands and in a lesser degree in the luminal epithelial cells as a result of DHEA administration (Fig. 3C-H). Parallel uterine tissues were stained by immunofluorescence with a specific Golgi marker antibody (TGN38). The analysis showed a similar distribution pattern for

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the Golgi marker protein and NHERF1 (Fig. 3I-J). For each gland, we classified the expression of NHERF1 in the apical/cytoplasmic or supranuclear/golgi area, and we counted the total number of glands/section in consecutive tissue sections of control and treated rats. We noticed that the staining was homogeneous for all the cells in the gland, and that they were all in the apical/citoplasmic or supranuclear/golgi area. The average amount of glands per section in the 7-day protocol was similar between control group 4.5 ± 1.5 and DHEA-treated 4.5 ± 1.8. The average gland number in the 20-day protocol was slightly higher in the control group 7.7 ± 0.7 compared to the DHEA-treated group 5.0 ± 0.5 ($p<0.017$). In the 7-day protocol, all of the glands of rats treated with DHEA showed a clear accumulation on NHERF1 in the "Golgi area", whereas all glands from control animals showed NHERF1 in the apical and cytoplasm region. In the 20-day protocol, all uterine sections showed at least one gland with "Golgi area staining" and 67.2 percent of all DHEA glands analyzed showed staining in the "Golgi area", whereas all the controls displayed the staining at the apical/cytoplasm membrane (Table 1).

DISCUSSION

Here we studied the effect of DHEA administration to immature Wistar rats for 7 and 20 days, and we observed morphological and cellular alterations consistent with a PCOS condition as early as 7 days of DHEA injection. Our results were in agreement with previous reports, a study conducted in 400 rats, where 6mg/100g of DHEA was administrated for 10, 15 and 20 days found that the majority of the rats has no corpus luteum and have developed follicular cyst as early as 10 days of DHEA.[28] We also detected the development of dysplastic lesions in the uterine epithelium after 20 days of treatment that were consistent with previous reports that showed a detrimental effect of DHEA in mouse uterus.[29] Failure of implantation linked with

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defective endometrial receptivity has been observed in women with PCOS, but the molecular causes remain unknown.[30] Recent studies, in rats and humans, strongly support a role of NHERF1 in uterine receptivity revealing the importance of NHERF1 subcellular localization in cytoskeleton changes at the implantation site.[31,32]

Uterine glands have a crucial role in uterus receptivity for embryo implantation and nutrition, and they also have an important role in the development of the fetus and placenta. The secretion of macromolecules by the endometrial glands is called "histiotroph" and constitutes the initial nutrition of the conceptus and fetus in humans, rats and other species before the placenta is developed and the fetal tissues receive nutrients from the maternal blood.[33] Several macromolecules have been identified in the histiotroph of humans and rodents such as Glycogen, Glycodelin A, Mucin 1 (MUC-1) and a number of cytokines.[34] For instance, Glicodelin A levels in uterine fluid is reduced in infertile women suggesting a role of this protein in uterus receptivity.[35] In this context, understanding the regulation of the uterine gland's secretion is the key to understanding the normal physiology of the glands and the alterations found in pathological conditions produced by genetic disorders or environmental factors. The administration of DHEA in 22 day-old Wistar rats showed a strong effect in the cellular distribution of NHERF1 in glandular and certain areas of luminal epithelial cells of the uterus. NHERF1 accumulates in the "Golgi apparatus area" in virtually all of the glands in the 7-day protocol and the majority of the glands in the 20-day protocol. At present, there are no reports about the direct action of DHEA in the Golgi apparatus of rat endometrial glands or the effect of DHEA in NHERF1 expression or cell distribution. It was known that NHERF1 plays an important role in the endocytic pathway participating in protein sorting and recycling as it was

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described for the CFTR and 2-adrenergic receptor.[36,37] To date, the sorting of signals from the Golgi apparatus and transport of proteins to the basolateral membrane are more conserved and better characterized than the apical ones.[38] The mechanism of protein oligomerization as delivery to the apical membrane was only previously described for lipids, so the apical protein sorting of Podocalyxin/Gp135 that was mediated by an adaptor protein such as NHERF1 opened a new paradigm in the mechanism involved in this process.[39] Several studies in different species showed the effect of estradiol-17β in the organelles of uterine epithelia and uterine glands. In ovariectomized sheep uterine glands, the treatment with estradiol for two days induce a significant increase in cell height and morphological alterations in the Golgi apparatus and the rough endoplasmic reticulum (RER). The Golgi apparatus and the RER were enlarged, with numerous vacuoles associated to the trans-Golgi network indicated an increase protein synthesis and secretory activity.[40] These changes in uterine glands had been observed in another species such as cat, dogs, human and non human primates.[41-43] The accumulation of NHERF1 at the "Golgi area" that we found as a response to DHEA administration, could affect the sorting of proteins to the apical membrane and gland secretion. Also, the diminished amount of NHERF1 at the apical membrane could have and effect in the organization of the apical epithelial compartment. Further studies need to be undertaken to determine to what extent NHERF1 functions in the Golgi machinery.

DHEA is and inactive hormonal precursor that exerts its actions through conversion to androgens or estrogens in peripheral tissues. To date, no specific receptor for DHEA has been characterized. The 17β hydroxysteroid dehydrogenase enzyme (17β-HSD) converts DHEA to androstenediol which is the substrate of the 3β-HSD to produce testosterone. The final step in the

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conversion of testosterone to estrogens is catalyzed by the aromatase enzyme that is present in several tissues in addition to the gonads and the endometrium. The specific balance determining the synthesis of androgens or estrogens from DHEA depends on the expression of steroidogenic enzymes, so the hormonal context within each cell will determine the ultimate activation of an androgen or estrogen receptor. Recent reports showed that women with high levels of DHEA in blood, such as those observed in PCOS, showed an increased androstenediol synthesis and higher expression of estrogen and androgen receptors in the endometrium. It was also showed that, the uterine androstenediol exert estrogenic instead of androgenic actions on the endometrial cells of PCOS women.[44,45] Within this context, the changes in NHERF1 subcellular distribution are more likely a result of estrogenic action instead a direct effect of DHEA or testosterone. As it was mentioned before, NHERF1 protein synthesis is stimulated by estrogens but not by testosterone, and changes in protein synthesizing organelles such as the Golgi apparatus and RER have been observed in endometrium in response to estrogen stimulation.

In summary, the effect of NHERF1 subcellular distribution may have a substantial effect in the uterus receptivity from two aspects: possible changes in normal glandular secretion and alteration of the membrane ultrastructure and remodeling during the first attachment of the trophoblast. Further study remains to determine whether NHERF1 could be used as a marker or early indicator of hormone imbalance and hiperandrogenism from endogenous causes or external endocrine disruptor chemicals.

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FIG. 1. Ovarian and uterine histology of control and DHEA-injected rats. The images show Hematoxilin and Eosin (H&E) stained section of 10% formalin fixed tissues. (A) Ovary from control vehicle-treated rats (7-days), showing secondary (preantral), tertiary (antral) and preovulatory (graafian) follicles. (B) Ovary from 7-days DHEA-treated rats with an increased number of secondary and atresic follicles (Af). (C). Ovary from 20-day control rat showing the presence of corpus luteum (Cl). (D) Ovary from 43 day-old rat treated with DHEA for 20 days. Notice the presence of large cystic follicle (Cf) with a diminished layer of granulose cells and cellular debris (Cd) in the lumen and no corpus luteum. (E) Control uterine epithelium. (F) DHEA- treated epithelium showing the alteration in epithelial morphology and increased epithelial height (H). (G) Altered morphology of epithelium from 20-day DHEA treated rats showing slight dysplastic morphology with vacuolization (V) of the endometrial cells hyperchromatism, hyperplasia and increased layer of cells in the epithelia. (H) Hyperplasic and dysplastic (D) uterine epithelium from a 20-day DHEA-treated rat. Original magnifications: X100 (A-D), and X400 (E-H).

FIG. 2. DHEA administration induced changes in ovarian and uterus weight and morphology. (A) Enlarged uterus diameter in rats injected with DHEA for 7 days (control, n=3; DHEA, n=9) ($p=0.0041$). (B) Increase in epithelial cell height in the luminal epithelial uterine cells after DHEA administration for 7 days (control, n=3; DHEA, n=6) ($p< 0.05$) (C) The ovaries of the 20-day protocol with DHEA were smaller and their weight was significantly decreased compared to controls (control, n=4; DHEA, n=4) ($p=0.0094$). (D) The uterine weight was significantly higher in DHEA-injected rats (20-day protocol) (control, n=4; DHEA, n=4) ($p<0.05$). (E) The diameter

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of the largest follicle was measured in duplicated slides of each ovary in DHEA (20 day-protocol) and control rats. Consistent with the presence of ovarian cysts, the diameter of the largest follicle was larger in the DHEA-treated rats (control, n=3; DHEA, n=4) ($p< 0.04$). The results are expressed as mean±SD.

FIG. 3. DHEA administration induces NHERF1 accumulation in the "Golgi area" of glandular uterine epithelial cells. NHERF1 immunohistochemistry analysis of liver (A) and kidney (B) from control rats showing the specific staining (arrows) in bile canaliculi (Bc) of the liver and renal proximal tubule (Pt) of the kidney, and the negative staining of the glomerulus (G) of the kidney (head arrows). (C) Control luminal epithelium of the uterus showing the normal apical (Ap) and slightly cytoplasmic NHERF1 localization at the cell membrane. (D) Epithelium from 20-day DHEA-treated rat uterus displaying a different NHERF1 expression pattern in enlarged epithelial cells with a reduction of apical staining and localization of the protein in the supranuclear/Golgi area (Ga). (E) Glandular epithelium from control rats (20-day vehicle) with positive NHERF1 staining in the apical/cytoplasmic cell compartment. (F) Cellular redistribution of NHERF1 in glandular epithelium (Ge) of 20-day DHEA treated rats showing staining in "Golgi area" (Ga) of the cells. (G) Section of uterus control (7-day vehicle) showing apical staining (Ap) and (H) 7-day DHEA treated uterine gland showing a clear accumulation of NHERF in the supranuclear/Golgi area (Ga) after 7 days of DHEA administration. The staining in G and H was developed at shorter time by design in order to have a better visualization of the label. (I and J) Immunofluorescence labeling of the Golgi apparatus of the luminal epithelium (I) and endometrial glands (J) using a specific antibody (for details see Materials and Methods). Notice the staining in the "supranuclear area" (arrows) similar to the one observed with the anti-NHERF1 antibody. Original magnification (A-J) X1000.

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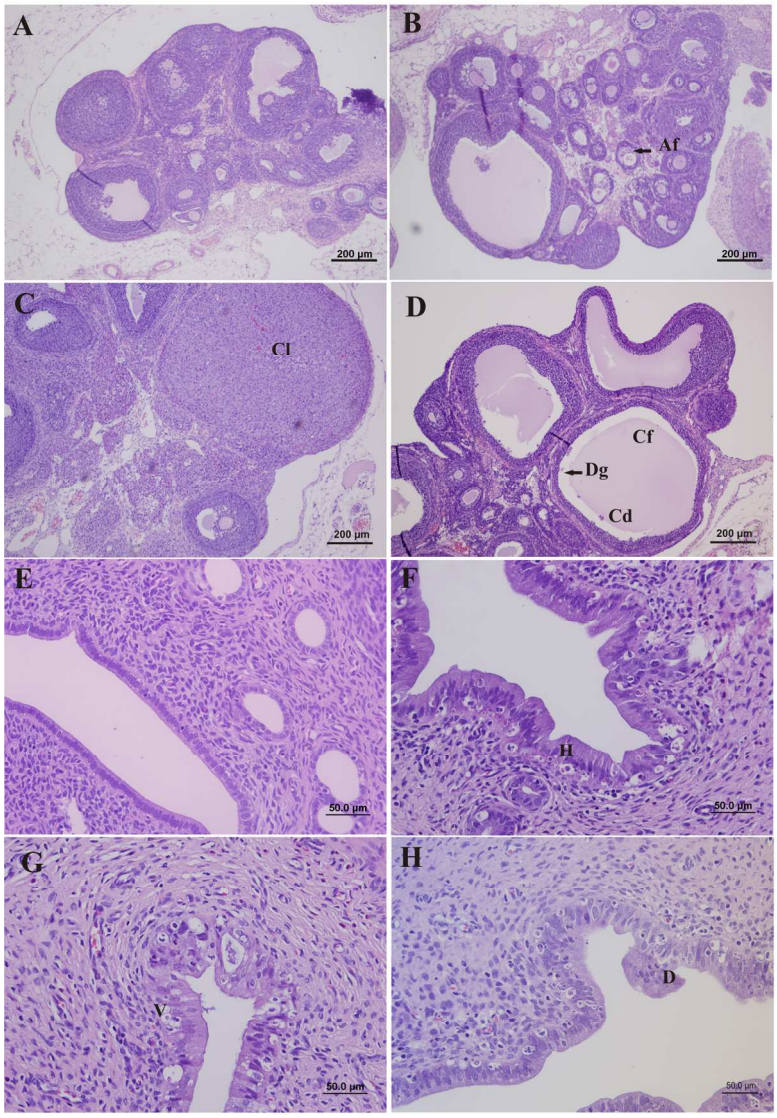
Figure 1



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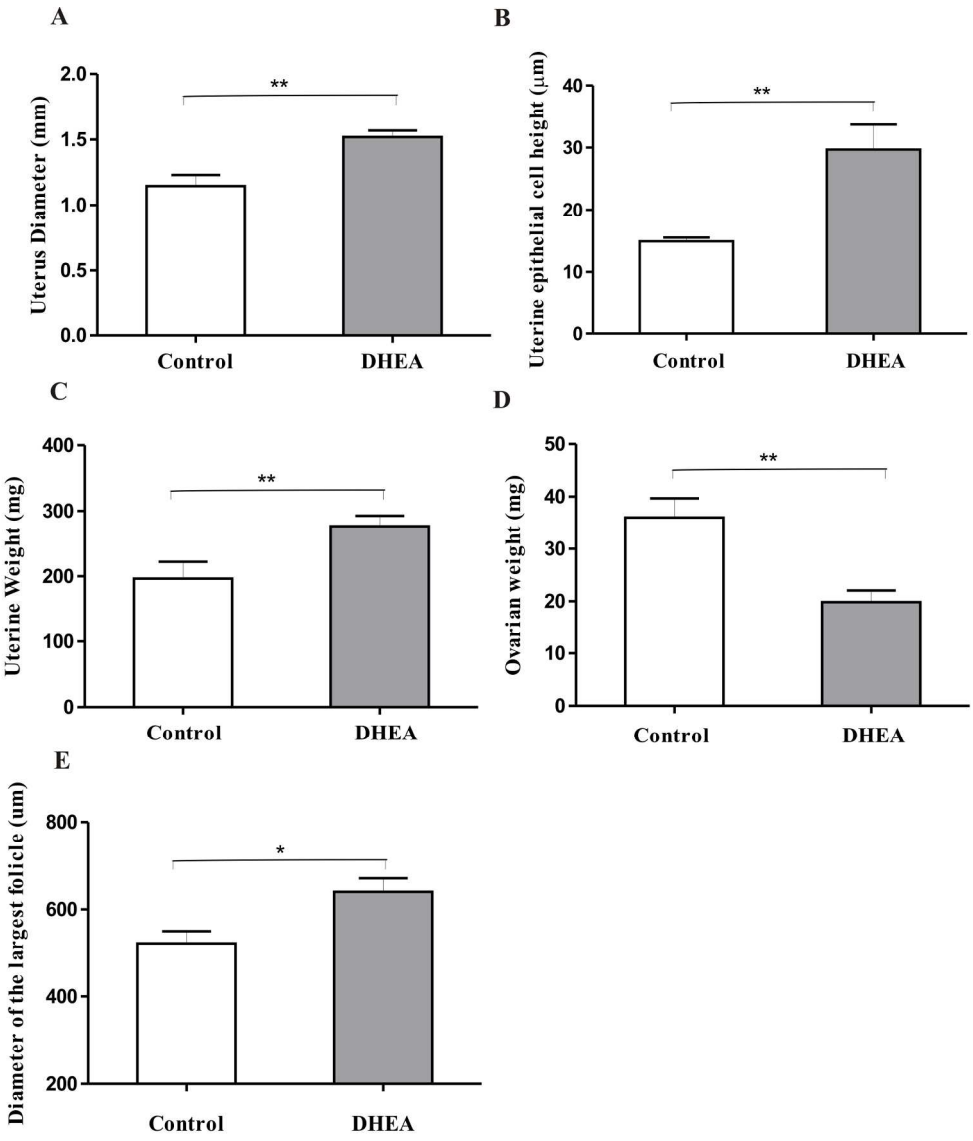

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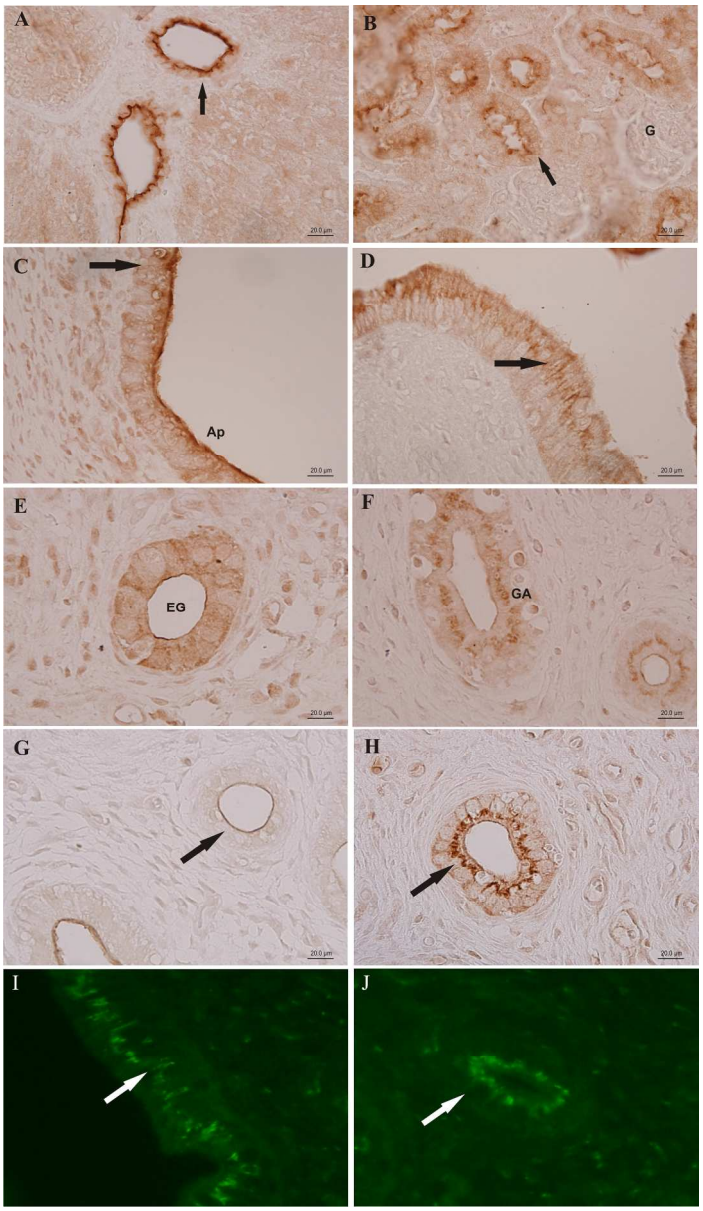

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Table 1. Cellular distribution of NHERF1 expression in endometrial glands of control and DHEA treated Wistar rats

Experimental Groups	Apical/Cytoplasm (%) glands	Apical/Golgi Area (%) Glands	Total Fields Analyzed
Vehicle 7 days	100	-	47* (n=5)
DHEA 7 days	-	100	69 (n=7)
Vehicle 20 days	100	-	98 (n=4)
DHEA 20 days	32.8	67.2	94 (n=5)

*The total number of fields corresponds to the gland number counted in 2-4 consecutives slides/rat.

Immunohistochemistry analysis of NHERF1 expression in endometrial glands of rats injected with vehicle or DHEA for 7 and 20 days. For each gland, we classified the expression of NHERF1 in the apical/cytoplasmic or supranuclear/golgi area, and we counted the total number of glands/section in consecutive tissue sections of control and treated rats. In the 7-day protocol, all of the glands of rats treated with DHEA showed a clear accumulation on NHERF1 in the "Golgi area," whereas all glands from control animals showed NHERF1 in the apical and cytoplasm region. In the 20-day protocol, all uterine sections showed at least one gland with "Golgi area staining" and 67.2 percent of all DHEA glands analyzed showed staining in the "Golgi area," whereas all the controls displayed the staining at the apical/cytoplasm membrane.

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