Expression profiling of lipocalin-2 and 24p3 receptor in murine gonads at different developmental stages

ELSA DE LA CHESNAYE[1], LETICIA MANUEL-APOLINAR[2], LETICIA DAMASIO[2], ALEIDA OLIVARES[3], MIGUEL ANGEL PALOMINO[1], ISIS SANTOS[3] and JUAN PABLO MÉNDEZ[4]

[1]Cardiovascular and Metabolic Diseases Research Unit, and [2]Endocrine Research Unit, National Medical Center, Mexican Social Security Institute, México City 06720; [3]Research Unit in Reproductive Medicine, Gyneco-Obstetrics Hospital, Mexican Social Security Institute, México City 01090; [4]Peripheral Obesity Research Unit, Faculty of Medicine, National Autonomous University of Mexico, México City 14000, México

Abstract. Numerous clinical studies have reported the association between high circulating levels of lipocalin-2 (LCN2) and metabolic diseases. However, only few studies have addressed sexually dimorphic, either in its circulating concentration or in its expression in other organs. To the best of our knowledge, LCN2 and the 24p3 receptor (24p3R), have not been identified in gonads; therefore, the present study analyzed their mRNA expression profile and cellular localization in gonads collected from fetal rats at 21 days post coitum, as well as from neonatal rats at 0, 2, 4, 6, 12, 20 and 30 postnatal days. Semiquantitative polymerase chain reaction and immunohistochemical assays revealed that the LCN2 mRNA during perinatal and pre-pubertal stages presented a sex-specific expression pattern, being higher in ovaries than in testes collected at these stages. Furthermore, the mRNA levels of the long and short isoforms of the 24p3R (507 and 350 bp, respectively), were lower in female gonads from postnatal day 0 onwards in comparison with the levels observed in males, but before birth, the short isoform of the 24p3R was higher in ovaries than in testes. In addition, in females, the abundance of mRNA of this isoform was drastically diminished at 24 h after birth. Furthermore, this specific expression profile of LCN2 and 24p3R at perinatal and prepubertal stages coincides with events of cellular proliferation and apoptosis within both gonads. Immunohistochemical assays revealed that in ovaries, LCN2 and 24p3R are present in germinal and somatic cells of follicles, while in testes, this adipokine and its receptor are only located in germinal cells. These findings suggest that in murine gonads, LCN2/24p3R signaling may be involved either in cell proliferation or cell death driven by gonadotropin-independent or -dependent mechanisms.

Introduction

Currently, adipose tissue is considered an endocrine organ which synthesizes a variety of adipokines and chemokines that are released into the circulation to exert their effects on various tissues.

Similar to sex-specific differences in body fat distribution, differential plasma concentrations of various adipokines have been reported. It is well known that from the beginning of puberty, sex-specific differences in plasma levels of leptin, adiponectin and ghrelin prevail, with these values being higher in women than in men (1,2).

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated LCN, has drawn the attention of numerous researchers due to its implication in metabolic pathologies (3). LCN2 is a member of the LCN superfamily comprised of small secreted proteins, characterized by the presence of three conserved motifs, constituting a single eight-stranded anti-parallel beta-barrel similar to a calyx that has the ability to bind organic ligands, specific cell surface receptors or to form complexes with soluble macromolecules. These three specific features confer a vast functional diversity. Thus, LCNs are involved in different roles, including retinol transport, cryptic coloration, olfaction, pheromone transport and enzymatic synthesis of prostaglandins. They are also implicated in the regulation of the immune response and cell homeostasis (4).

The human LCN2 gene is located on chromosome 9q34 and comprises a 3,696-bp coding region, which contains seven exons and six introns (5). The corresponding protein is a 25-kDa secreted glycoprotein, initially identified in neutrophils covalently linked to matrix metalloproteinase (MMP)-9. It is also present as a 46-kDa disulphide-linked homodimer and a 135-kDa disulphide-linked heterodimer (6). This adipokine

Correspondence to: Dr Elsa de la Chesnaye, Cardiovascular and Metabolic Diseases Research Unit, Mexican Social Security Institute, 330 Cuauhtémoc Avenue, Cuauhtémoc, México City 06720, México
E-mail: edelachesnaye@yahoo.com

Dr Juan Pablo Méndez, Peripheral Obesity Research Unit, Faculty of Medicine, National Autonomous University of Mexico, 15 Vasco de Quiroga, México City 14000, México
E-mail: jpmb@unam.mx

binds to a specific cell surface receptor, the 24p3 receptor (24p3R), in order to be internalized into the cell to regulate various physiological processes, including iron delivery or uptake, and cellular apoptosis (7). The corresponding rat gene, designated as LCN2, 24p3 or Sip24, is located on chromosome 3p12, and encodes an mRNA of 596 bp with only one exon and a 24-kDa protein with 198 amino acids (8).

Clinical studies have reported an association of high circulating levels of LCN2 with obesity and insulin resistance (9). Conversely, others have demonstrated a reduction of LCN2 levels in obese and non-obese diabetic individuals, or in women with polycystic ovarian syndrome (10,11); however, in these two studies, cardiac alterations were present in the patients analyzed. Regarding the latter, it is worth mentioning that this adipokine is an important modulator of inflammation (12). Therefore, it may be suggested that this ambivalence in the reduction or increment of LCN2 levels within cardiometabolic alterations depends on the level of inflammation due to the disease stage and whether or not such disease has been controlled. In this context, a previous study by our group reported a statistically significant decrease in plasma levels of LCN2 in Mexican patients with type 2 diabetes mellitus in comparison with those in control subjects (13). Another previous study by our group reported sex-associated differences in LCN2 plasma levels in healthy individuals (14). However, few studies have assessed the possibility of sex-specific differences in the levels of LCN2, either in its circulating concentration or its expression in other organs (13-15). To the best of our knowledge, LCN2 has not been previously determined within the gonads.

In order to identify differentially expressed genes in the neonatal murine ovary, a previous study by our group employed a DNA microarray to interrogate the mouse genome, identifying the LCN2 gene within a cluster of DNA sequences whose expression profiles were increased during the first 4 postnatal days, when folliculogenesis takes place (16). This result, as well as the fact that the murine LCN2 gene contains estrogen recognition sites within its promoter region (17), suggested the presence of LCN2 protein in the gonads and its regulation by hormones, and therefore its sex-specific differential expression.

Taking this into account, the present study analyzed the mRNA expression levels of LCN2 and its receptor 24p3R, as well as the respective protein profiles in ovaries and testes of wild-type rats.

Materials and methods

Animals. Animal experiments were performed using Sprague Dawley rats obtained from an inbred colony at the National Medical Center, Mexican Social Security Institute (México City, México). A total of 10 female and 10 male rats (3 months old and 200-250 g) were housed under a 12-h light/dark cycle, temperature of 21±2°C and 60% humidity, and were given free access to rodent chow and tap water (5008 Formulab Diet; PMI Nutrition International, Brentwood, MO, USA). The experimental protocol was approved by the Research Committees of the National Medical Center and the National Autonomous University of Mexico (México City, México; approval no. UNAM-003-2013), and was performed in accordance with the American Association for Accreditation of Laboratory Care and National Institutes of Health guidelines.

Following an adaptation period, each of ten female rats was mated with a male. The presence of vaginal plugs was examined the morning after mating. Confirmation of a vaginal plug was designated as postcoitum day 1 (1 dpc). Likewise, the day of birth was designated as postnatal day 0 (0 dpn). Following birth, offspring from the 10 pregnant rats were weighed and, in order to assure adequate and standardized nutrition until weaning, litter sizes were normalized to eight pups per litter (4 females and 4 males). The remaining pups of each litter were sacrificed by decapitation immediately following birth. Maternal animals were fed *ad libitum* during lactation. Each litter was weighed weekly.

A total of 3 pregnant rats were sacrificed at 21 dpc, and the ovaries and testes from the 12 female and 12 male pups were collected. Ovaries and testes from 4 females and 4 males pups at each of the following time points: 0, 2, 4, 6, 12, 20 and 30 dpn, were also collected immediately after pups were sacrificed by decapitation. Upon collection, gonadal tissue was either frozen on dry ice for RNA isolation or fixed for immunohistochemistry.

RNA isolation. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. In brief, the tissue was homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH, USA), and the aqueous and organic phases were separated by addition of one volume of bromo-3-chloropropane (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), followed by centrifugation at 13,800 x g for 15 min at 4°C. Thereafter, 70% ethanol (350 μl) was added to each sample, which was then applied to an RNeasy minicolumn (Qiagen), followed by washing by centrifugation at 735 x g for 2 min at room temperature with buffers containing guanidine and ethanol. To elute the RNA, 30 μl RNase-free water was added directly onto the silica-gel membrane of each of the columns, which were then centrifuged for 1 min at 13,800 x g at room temperature. The RNA was quantified by measuring the absorbance at 260 nm and stored at -85°C until use. The quality of each RNA sample was assessed on a 2% formaldehyde denaturing agarose gel.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). To assess the relative expression of LCN2 and 24p3R mRNA in ovaries and testes collected at the different stages mentioned above, total RNA from all samples was first reverse-transcribed using the Superscript First-Strand kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. All reactions were performed in a total volume of 20 μl. Initially, 200 ng total RNA, isolated from the gonads, was annealed at 65°C for 5 min in the presence of 0.5 μg oligo (dT) 12-18 primer (0.5 μg/μl) and 1 μl dinucleoside triphosphate (dNTP) cocktail (10 mM). The annealed RNA-primer samples were incubated for 1 h at 42°C with RT buffer (10X), MgCl$_2$ (25 mM), RNaseOUT (40 U/μl) and 50 U Superscript II reverse transcriptase (50 U/μl). Reactions were terminated by incubation at 70°C for 15 min, followed by incubation at 37°C for 20 min with 2 U of *Escherichia coli* RNase H (2 U/μl).

PCR amplification was performed using the QuantumRNA 18S Internal Standard kit (Ambion; Thermo Fisher Scientific, Inc.) in a total volume of 25 μl, containing 2.5 μl 10X

PCR buffer, dNTPs (0.1 mM) and 0.15 μl Taq polymerase (5 U/μl; HotStar Taq; Qiagen) plus 2.5 μl of a mixture of 18S primers/competimers at a ratio of 3:7 and 1 μl complementary (c)DNA template annealed to 10 pmol of LCN2- and 24p3R-specific primers (Table I).

The PCR conditions used for LCN2, 24p3R and 18s amplification were 5 min at 94°C to activate the HotStar Taq enzyme, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C and 1 min of extension at 72°C, followed by a 10-min final extension at 72°C. In all PCR experiments, a reaction with all PCR components with the exception of DNA was used as a negative control. Equal volumes of PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. Subsequently, the gels were scanned and the images were quantitated by densitometry using image analysis software (Quantity One; version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

In order to verify LCN2 and 24p3R cDNA amplification, purified samples were sequenced on an Applied Biosystems DNA Sequencer model 377, using the Big Dye™ Terminator Sequencing Ready Reaction kit version 3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sequencing was performed following the protocol supplied by the manufacturer. Sequencing results were compared against the GenBank sequence database by means of the Basic Local Alignment Search Tool algorithm of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Western blot analysis. To determine the protein levels of LCN2 and 24p3R in rat gonadal tissue, total protein extracts were obtained from ovaries, testes and kidneys of adult wild-type rats by homogenization using a low-ionic-force buffer containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM), $MgCl_2$ (1.5 mM) and KCl (10 mM), supplemented with aprotinin (10 μg/ml), penylmethane sulfonylfluoride (1 mM), dithiothreitol (0.5 mM) and the protease-inhibitor 1,10-phenantroline (10 mM; Sigma-Aldrich; Merck KGaA). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc.) and thereafter, proteins were denatured at 37°C for 30 min, and 50 μg/well of denatured protein was electrophoresed on a 10% Tris-Glycine SDS-PAGE gel, transferred onto an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA) and blocked at 4°C for 1 h, with freshly prepared solution of 5% bovine serum albumin (BSA; Bio-Rad Laboratories, Inc.) in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween 20. The membrane was immunoblotted overnight at 4°C with gentle agitation using either a rat polyclonal antibody against LCN2 or against 24p3R (cat. no. ab41105 and ab124506, respectively; Abcam, Cambridge, MA, USA) diluted at 1:200, followed by incubation with an anti-goat horseradish peroxidase-conjugated immunoglobulin (Ig)G (1:15,000 dilution; cat. no. 305035003; Jackson Immuno-Research, West Grove, PA, USA) for 1 h at room temperature. After stripping the membrane for 2 h at 55°C in a 62.5 mM Tris-HCl buffer (pH 6.8) containing 100 mM 2-mercaptoethanol and 2% SDS, the membrane was re-blocked and re-probed using an anti-GAPDH mouse monoclonal antibody (cat. no. MAB374; Merck KGaA, Darmstadt, Germany) at 1:10,000 dilution overnight at room temperature and detected with an anti-mouse HRP-conjugated IgG (cat.

no. 115035003; Jackson Immuno-Research) at a dilution of 1:15,000 at room temperature for 2 h. The reaction was visualized after 6 min of exposure with enhanced chemiluminescence reagents (Perkin Elmer; Thermo-Fisher Scientific, Inc.). 293 cells (American Type Culture Collection, Manassas, VA, USA) were used as a negative control for LCN2 signaling. Protein extracts from 293 cells were obtained following the culturing of the cells in 75 cm^2 culture plates (Corning Incorporated, Corning, NY, USA) containing high glucose Dulbecco's modified Eagle medium, 5% fetal bovine serum, 2.0 mM L-glutamine, 50 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in 5% CO_2 at 37°C for 24 h.

Immunohistochemistry. In order to determine LCN2 and 24p3R protein signaling within gonads of wild-type rats, 5-μm sections of formalin-fixed, paraffin-embedded gonadal samples collected from 30-day-old rats were obtained and mounted on glass slides previously coated with poly-L-lysine, and then deparaffinized and rehydrated in a graded series of ethanols (100, 90, 70 and 30% and water). Sections were then microwave-heated with antigen retrieval solution (Vector Laboratories, Burlingame, CA, USA), rinsed in 1X PBS (pH 7.4) and incubated for 30 min in 3% H_2O_2 in methanol to inactivate endogenous peroxidase, and subsequently blocked with 10% BSA in 1X PBS for 30 min. Tissues were then incubated with either primary rat anti-LCN2 or rat anti-24p3R antibody (dilution, 1:150; Abcam) at 4°C overnight. Sections were washed in PBS, incubated at room temperature for 2 h with the Mouse/Rabbit Immunodetector HRP/diaminobenzidine (DAB; Bio SB, Inc., Goleta, CA, USA) and washed with 1X PBS. The peroxidase reaction was developed with DAB and H_2O_2 generating a brown precipitate. Finally, slides were counterstained with hematoxylin, dehydrated and mounted with synthetic resin. Sections of uterus collected from the same wild-type female rats were used as a positive control. The negative control was treated with BSA in PBS instead of primary antibody.

Statistical analysis. Values are expressed as the mean ± standard deviation. Comparisons between two groups were made by an unpaired two-tailed Student's t-test. Comparisons between the mRNA expression levels in ovaries and testicles were made by two-way analysis of variance followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism 4 for Windows (GraphPad Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. To obtain significant results 80 animals were used with 8 per stage.

Results

LCN2 mRNA expression profiling in rat gonads. To determine LNC2 mRNA expression profiling during ovarian and testicular development in wild-type rats, RT-PCR using a set of primers that amplified a 592-bp fragment of the LCN2 sequence (Table I) was performed. As presented in Fig. 1, in rat ovaries, the relative expression of LCN2 mRNA was abundant at 21 dpc, but significantly decreased by ~50% at <24 h after birth (P<0.05), increased again at 12 and 20 dpn and stayed at this relatively high level until 30 dpn, when
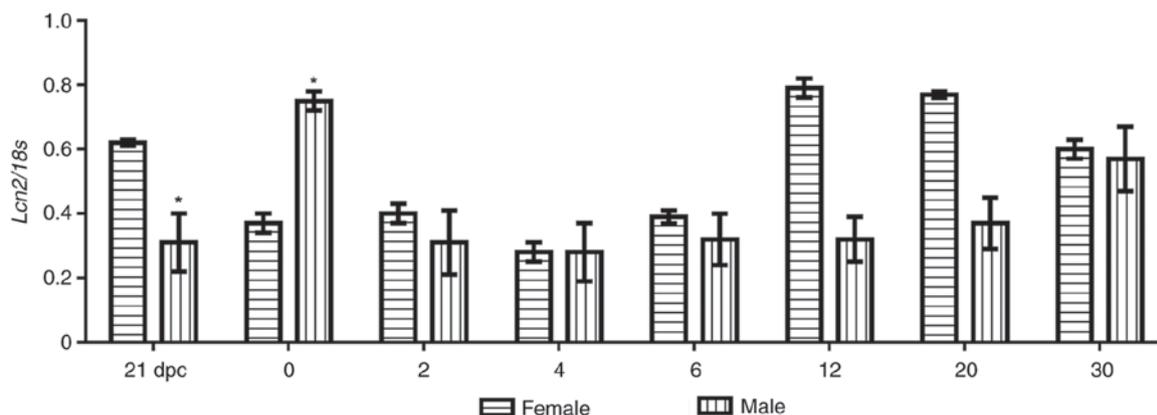
Figure 1. Lipocalin-2 mRNA is expressed in rat gonads. In ovaries, its abundance increases transiently at 21 dpc, when follicular assembly is about to take place, and later on at prepubertal and pubertal stages when the onset of gonadotropic stimulus occurs. Conversely, in testicles LCN2 mRNA levels are low at the same age, but its abundance increases hours after birth only to diminish two days later and increase again at 30 dpn. The graph depicts the quantification of the relative density of LCN2 mRNA from gels. The constitutively expressed 18s ribosomal RNA was used as the normalizing unit. The y-axis indicates the expression levels of LCN2 relative to 18s ribosomal RNA and the x-axis indicates age in dpn unless otherwise indicated. Values are expressed as the mean ± standard deviation. *P<0.05 male vs. female. Dpc, days postcoitum; dpn, days postnatal; LCN2, lipocalin-2.

Table I. Primers complementary to LCN2, 24p3R and 18s genomic sequences used for complementary DNA amplification.

Gene name /direction	Sequence (5'-3')	Product length (bp)
LCN2		
Forward	TCTCGATTCCGTCGGGTGGTGG	592
Reverse	CCTGGGTGTCCTGTGTCTG	
24p3R		
Forward	AGGACTGGGACTACAACGGA	507
Reverse	GTGCGGACTCCAGAAACAGA	
18s		
Forward	TCTCGATTCCGTCGGGTGGTGG	360
Reverse	CTTATGACCCGCACTTACTCG	

LCN2, lipocalin-2; 24p3R, 24p3 receptor. The 350 bp isoform was amplified by chance using the 507 bp pair of primers.
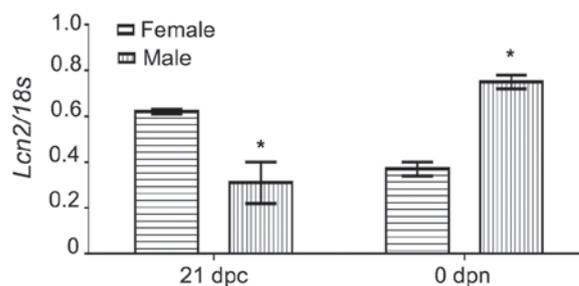

Figure 2. LCN2 mRNA presents a different pattern of expression between females and males at perinatal stages. Graph depicting the quantification of the changes in the gel. The y-axis indicates the expression levels of LCN2 relative to 18s ribosomal RNA. Values are expressed as the mean ± standard deviation. *P<0.05, female vs. male prior to and <24 h after birth. Dpc, days postcoitum; dpn, days postnatal; LCN2, lipocalin-2.

LCN2 mRNA expression decreased slightly. Conversely, in testicles, the LCN2 levels were low at 21 dpc, the mRNA was then abundant at <24 h postpartum (P<0.05), but from the second postnatal day onwards, its expression decreased again until 30 dpc, when LCN2 was expressed at approximately the same rate in the gonads from male and female animals (Fig. 1). These changes suggest that LCN2 mRNA expression in perinatal and pre-pubertal gonads exhibits a sex-specific pattern (Figs. 1 and 2). This also raised the question as to whether this specific pattern of expression is mediated via 24p3R. To address this, another RT-PCR assay was performed to amplify the 507-bp fragment of the 24p3R mRNA sequence, employing a specific set of primers (Table I). Electrophoresis of the PCR products revealed the presence of the intended 507-bp band, as well as a lower-size band (350 bp). The 507-bp fragment exhibited a constant relative expression throughout the stages analyzed, being much lower in the female samples than in the male ones (Fig. 3). Of note, only in perinatal ovarian samples,

the relative expression of the small fragment of 24p3R cDNA (350 bp) was similar to that of LCN2 at the same perinatal stage. In other words, as that of LCN2, the relative expression of 24p3R was high at 21 dpc and decreased hours after birth (P≤0.05; Fig. 4). As a positive control, a portion of the ubiquitous 18s gene (360 bp) obtained from the same cDNA samples was also amplified.

LCN2 protein identification by western blot analysis. In order to assess the presence of LCN2 and 24p3R proteins within gonadal tissue, protein extracts from ovaries and testes of wild-type adult rats were employed to perform a western blot assay using polyclonal antibodies against LCN2 and 24p3R, respectively. As presented in Fig. 5A, a 24-kDa band, which corresponds to the molecular weight of LCN2, was observed in each of the two protein samples. This signal was also identified in protein extracts isolated from kidneys of the same animals, where LCN2 synthesis has been demonstrated. In fact, high concentrations of LCN2 in urine and plasma are now considered as biological markers for acute kidney injury (18). Furthermore, the 24p3 receptor was detected within the male and female gonads. As displayed in Fig. 5B, western blot analysis of ovarian
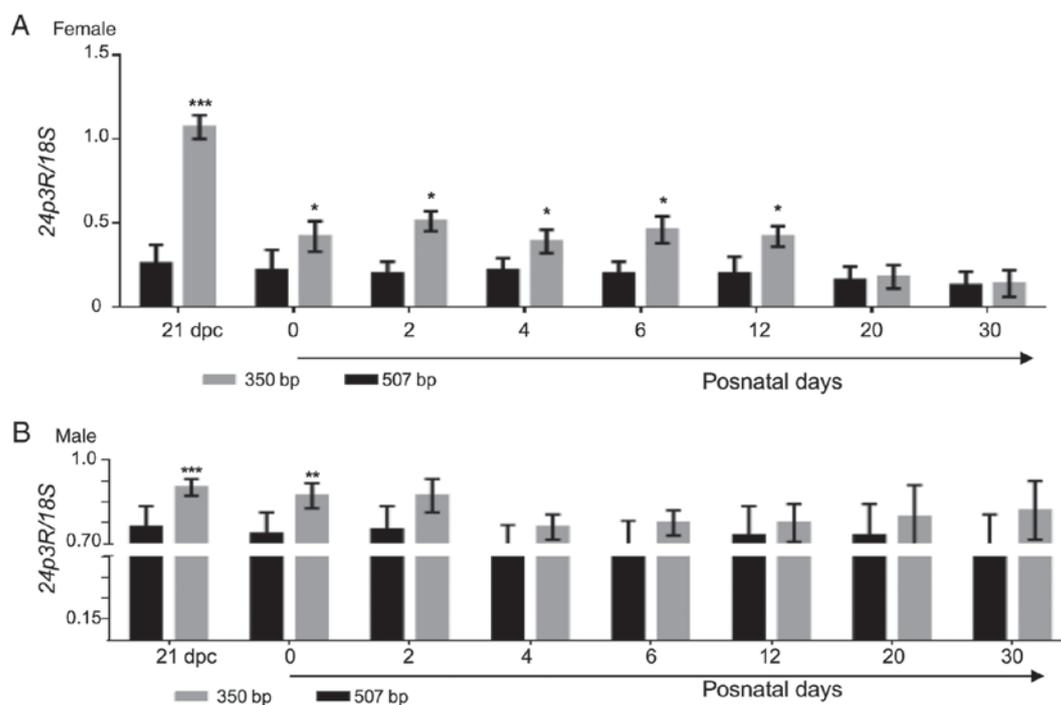
Figure 3. Relative expression of 24p3R in gonads. (A) In ovaries, long (507 bp) and short (350 bp) isoforms of the receptor are at a relatively low level throughout the stages analyzed. Only the short isoform of the 24p3R is at high levels at 21 dpc. (B) In testicles, the two isoforms present a consistent and higher abundance throughout the stages analyzed. The graphs depict the quantification of 24p3R mRNA determined from gel images by densitometry. The y-axis indicates the expression levels of 24p3R relative to 18s ribosomal RNA and the x-axis indicates age in dpn unless otherwise indicated. Values are expressed as the mean ± standard deviation. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ 350 bp vs. 507 bp isoform. Dpc, days postcoitum; dpn, days postnatal; 24p3R, 24p3 receptor.

protein extracts revealed a well-defined 57-kDa band, which corresponds to the molecular weight of the 24p3 receptor, which was also present at a lower intensity in the testicular and 293 protein samples. GAPDH was detected in the protein extracts of the three organs as a reference (Fig. 5A and B).

Cellular localization of LCN2 within gonadal tissue. The cellular localization of LCN2 and 24p3R within gonads from wild-type rats was determined by means of immunohistochemistry (Fig. 6). In sections of paraffin-embedded ovaries, LCN2 immunostaining of oocytes and granulosa cells of primordial and growing follicles, as well as in the zona pellucida and antrum of developed follicles, was observed (Fig. 6A and B). Regarding the 24p3 receptor, intense staining was observed in the zona pellucida of oocytes and in the antrum of the fully developed follicles (Fig. 6E).

In sections of paraffin-embedded testicles, LCN2 and 24p3R are present in germinal cells at different developmental stages, rather than in cells of epithelial origin (Fig. 6C and F).

Discussion

Adipokines comprise a vast number of molecules synthetized mainly in adipose tissue but present in other organs, which are involved in the regulation of numerous physiological processes, including immunity, appetite control and metabolism, as well as cardiovascular and reproductive function. Studies performed in knockout mice have provided evidence for the role of various adipokines in the regulation of the HPG axis (19). For instance, the participation of leptin in regulating the HPG axis is evidenced by the fact that leptin-deficient mice

are infertile (20). Leptin is localized to the pituitary gland, where it stimulates the production of gonadotrophin-releasing hormone (GnRH) through neurons possessing leptin receptors. In turn, GnRH causes the release of both the luteinizing hormone (LH) and the follicle stimulating hormone (FSH) that subsequently act on male and female gonads (21). In the same manner, adiponectin regulates reproductive function through the HPG axis. Its circulating concentration depends on GnRH and gonadotropins levels, which also vary according to the estrous cycle phase (22). Furthermore, LH and FSH modulate the expression of the adiponectin receptor 2 in ovarian granulosa cells in order to increase progesterone secretion (23).

To the best of our knowledge, the present study was the first to determine the expression profile of LCN2 or 24p3 and its receptor, 24p3R, in rat ovaries and testicles collected at different stages of gonadal development. LCN2 and 24p3R mRNA expression was observed in male and female gonads from 21 dpc onwards. In this context, the mRNA and protein expression of adiponectin, visfatin, resistin, chemerin and apelin have been identified in gonads of several species, leading to the conclusion that these adipokines are involved, through their specific receptors, in gonadal functions that are mostly gonadotropin-dependent, including germinal cell maturation, steroidogenesis or estradiol secretion (24-30). Nevertheless, none of these previous studies reported on the expression of any of the aforementioned adipokines during perinatal stages when gonadotropin-independent molecular mechanisms are taking place. In the present study, LCN2 and 24p3R mRNA expression was observed distinctively at these stages. Taking into account that LCN2 covalently binds to MMP-9 in order to prevent its degradation to allow for the modulation of cellular matrix remodeling (31), the mRNA
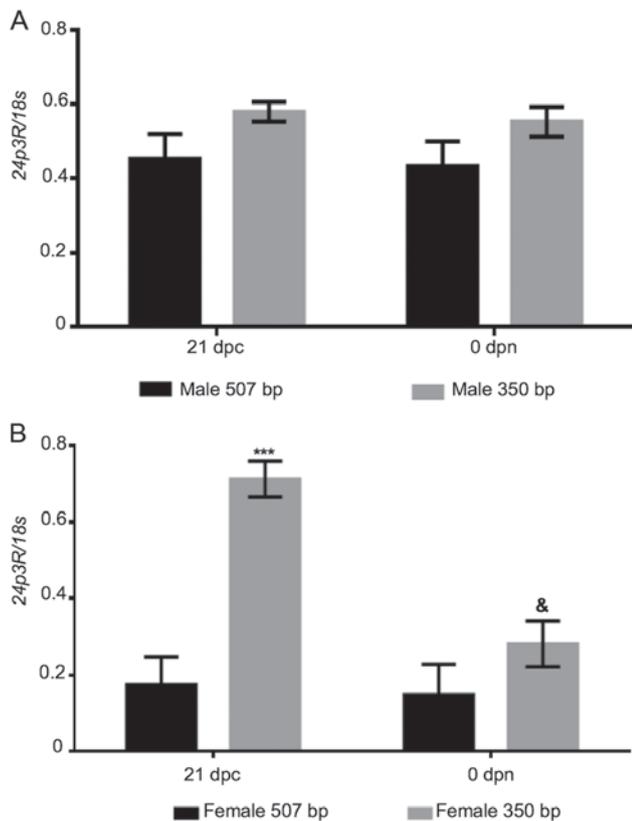
Figure 4. Relative expression of 24p3R mRNA during the perinatal period in the gonads of (A) male and (B) female rats. Graphs depict the quantified levels of 24p3R relative to 18s ribosomal RNA, determined by densitometry. In female rats, only the short isoform (350 bp) of the receptor displayed changes in mRNA expression in ovarian samples; such expression was abundant at 21 dpc and decreased hours after birth. The y-axis indicates the expression levels of 24p3R relative to 18s ribosomal RNA and the x-axis indicates age in dpn unless otherwise indicated. Values are expressed as the mean ± standard deviation. $^{\&}P<0.05$ 350 bp isoform at 21 dpc vs. <24 h after birth; $^{***}P<0.001$ 507 bp vs. 350 bp isoform at 21 dpc. Dpc, days postcoitum; dpn, days postnatal; 24p3R, 24p3 receptor.
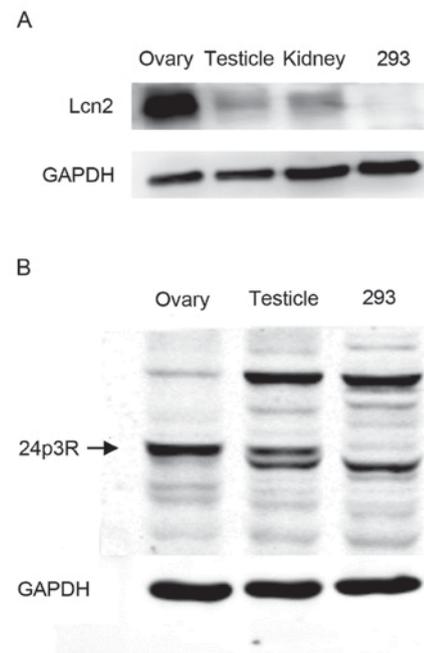

Figure 5. Identification of LCN2 and 24p3R protein by western blot assay. (A) Female and male gonads, as well as kidneys from adult rats express a ~24 kDa protein, similar in size to the LCN2 protein predicted by the LCN2 mRNA open reading frame. The lower panel depicts GAPDH identification, used as positive control for all samples. (B) 24p3R protein expression in ovaries and testes collected from adult rats. Ovarian and testicular protein extracts, as well as 293 cell samples express a ~57 kDa protein, similar in size to the 24p3R protein predicted by the 24p3R mRNA open reading frame. The lower panel depicts GAPDH identification. LCN2, lipocalin-2; 24p3R, 24p3 receptor.

expression profile of this metalloproteinase in male and female gonads was assessed, and a low and constitutive expression was observed in perinatal stages (data not shown). This is in agreement with a study by Light and Hammes (32), in which a detectable but extremely low MMP-9 mRNA expression was identified in primary granulosa cells of murine ovaries. Therefore, the present study focused on the 24p3 receptor, which, as mentioned above, exhibited a distinct pattern of expression in the perinatal period. LCN2 in conjunction with this receptor exerts or triggers different signaling pathways, including iron transport and regulation of various cellular processes, including cell differentiation and apoptosis (33,34). It is known that within male and female murine gonads, mitotic proliferation of germ cells is arrested by embryonic day 13.5, and in the case of the ovary, this is followed by progression through the prophase stage of the first meiotic division until around the time of birth, or in the case of the testis, a re-entry into the cell cycle of germ cells arrested in mitosis, in order to start spermatogenesis (35,36). In this regard, the present study also indicated that during these perinatal stages, the relative mRNA expression of LCN2 exhibits sex-specific differences and that at least in perinatal ovaries, the relative mRNA expression of the short isoform of 24p3R is identical to that of LCN2.

The latter, may be attributed to the fact that in the female gonad, the physiological processes performed at this stage are different from those that occur in the testicle. Therefore, LCN2/24p3R signaling may have different purposes within ovaries and testes. For instance, it is well known that during follicular assembly, which in murine gonads starts at perinatal stages, numerous oocytes are lost through apoptosis (37). The fact that binding of apo-LCN2 (iron-free LCN2) to the 24p3 receptor mediates intracellular iron depletion and subsequently leads to apoptosis (7), may indicate a specific role for LCN2/24p3R in apoptotic signaling in the perinatal ovary. Alternatively, iron-loaded LCN2 may be internalized through this receptor in order to increase intracellular iron levels and subsequently promote cell proliferation (38). Recent *in vitro* studies have demonstrated that another adipokine, apelin 13, promotes granulosa cell proliferation and apoptosis inhibition through the phosphoinositide-3 kinase/Akt signaling pathway (39). In addition, a protective role for apelin 13 against apoptosis has been demonstrated in brain and cardiac tissue (40,41); the latter scenario may also be alternatively considered for LCN2/24p3 in ovarian physiology. By contrast, in the male gonad, LCN2 mRNA increases hours after birth, but two days later, its expression diminishes by half. It is well established that apoptosis within the testis occurs also at a high rate, when the first spermatogenic cycle takes place at 10-30 days after birth. This cell death process is orchestrated primarily by a balance between pro-apoptotic proteins, including B-cell lymphoma 2 (Bcl-2)-associated X protein and the anti-apoptotic Bcl-2 protein family (42). Therefore, it is difficult to associate
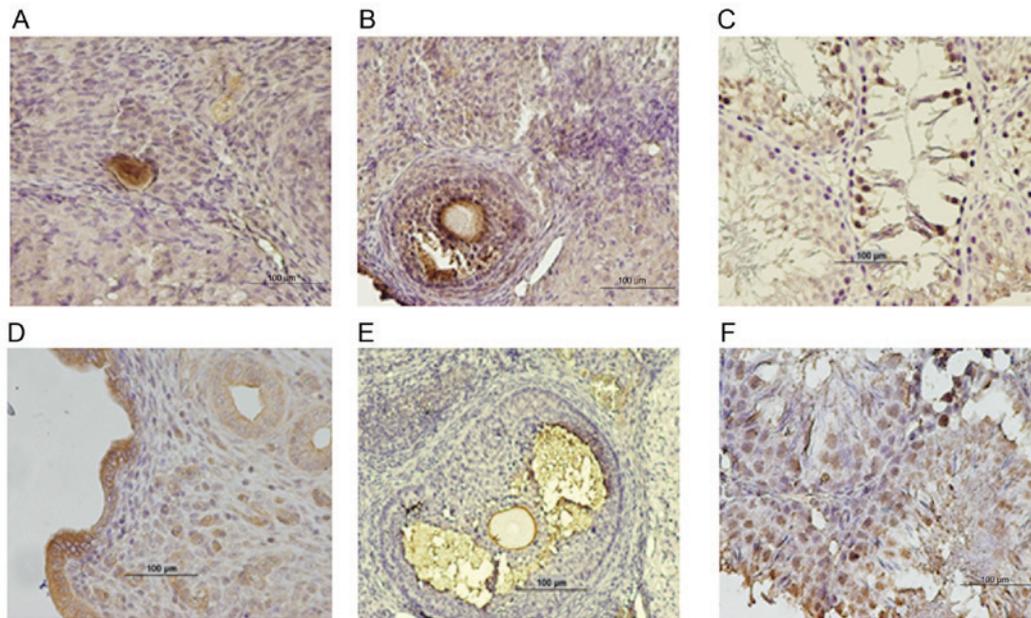
Figure 6. LCN2 and 24p3R expression in adult rat ovaries and testis. (A) Immunostaining for LCN2 in normal ovaries, with LCN2 located in oocytes surrounded by a single layer of epithelial granulosa cells. (B) Strong immunostaining for LCN2 in the cytoplasm of oocytes, as well as in the developing antrum of the respective follicle. A signal of less intensity was observed in follicular granulosa cells. Differences in signal intensity may be due to intrinsic experimental variability. (C) Immunopositivity for LCN2 in gametic cells of testicular samples. (D) Uterine tissue was used as positive control to LCN2 antibody according to the supplier's instructions. (E) Immunoreactivity of 24p3R in normal ovary, with staining observed in the oocytes' cytoplasm and in the zona pellucida, where a strong immunoreaction was detected. Immunopositivity for this receptor was also observed in the antrum of developing follicles. (F) The 24p3R signal is positive in spermatogonia of testicular samples (magnification, x40; scale bar, 100 μm). LCN2, lipocalin-2; 24p3R, 24p3 receptor.

an apoptotic process within testicles driven by LCN2 and 24p3 receptor occurring hours after birth with an increment of LCN2 mRNA expression. Thus, at this stage, the role of LCN2/24p3R in the procurement of male gonadal cell survival may also be considered.

Similarly, a distinct difference between the LCN2 mRNA profile of ovaries and testes was observed at postnatal days 12 and 20. The latter coincides with the expression of gonadotropin receptors and the onset of gonadotropin-dependent mechanisms. Various studies have localized chemerin, resistin and visfatin within somatic and germinal cells of the ovary. The first two adipokines are involved in the downregulation of ovarian steroidogenesis, mostly by inhibiting aromatase expression in granulosa cells (19,43). Visfatin appears to be involved in oocyte maturation, as its concentration in the follicular fluid has been associated with the number of mature oocytes (25).

In the present study, even though the relative expression of 24p3R in male and female gonads remained at a constant level at all stages, the mRNA abundance of the two isoforms observed in the ovary was not as much as that identified in the testis. A study published in 2003 by Burns et al (44), in which 24p3 is upregulated by 60-fold in FSH-null mice, suggested an inhibitory effect of FSH on the expression of this adipokine; this in turn may lead to the downregulation of 24p3R expression. In fact, a slight decrease in LCN2 and 24p3R mRNA levels at postnatal day 30 was observed, coinciding with the decrease of FSH. Regarding the male gonad, FSH acts in concert with sex hormones (testosterone and estradiol) to support male germ survival (42). Thus, the participation of LCN2/24p3R in different mechanisms within male and female gonads at this time of development should be considered. This sexually dimorphic expression of LCN2 was also reported by

Guo et al (17) in 2012, who observed higher levels of LCN2 in inguinal fat depots of female mice in comparison with the levels in males. Furthermore, they demonstrated an association between LCN2 and estrogen production and action in adipose tissue, which provides preliminary data on the association that may exist between this adipokine and the corresponding mechanisms, which are dependent on gonadotropic stimulus.

The localization of LCN2 and 24p3R to somatic and germ cells within the murine ovary observed in the present study is in accordance with the observations of studies performed by two other groups (19,45), which demonstrated that various adipokines and their corresponding receptors are involved in a coordinated crosstalk, which ensures proper follicular development. The latter study also suggests that in the ovary, LCN2/24p3R signaling may act in a paracrine and/or an autocrine manner. Of note, in the male gonad, LCN2 and its receptor are localized exclusively in germ cells of different maturational stages, indicating that as in the ovary, a coordinated communication between this adipokine and its receptor may also occur in the testes in order to achieve adequate germ cell development, but this communication only occurs in an autocrine way. At present, it is challenging to explain differences in the pattern of LCN2/24p3R localization between ovaries and testes due to the limited information available. In fact, studies focusing on the participation of adipokines in male gonadal function are scarce and the majority of them specifically address the association between metabolic diseases and poor quality, as well as low count or motility of sperm (19).

The present results may indicate that LCN2/24p3R signaling is involved in cell proliferation or apoptotic mechanisms within rat gonads and that such signaling is exerted in a sexually dimorphic pattern at different stages of gonadal development.

Even though the present study demonstrates the presence of LCN2 and 24p3R in rat gonads, it is limited in terms of not experimentally demonstrating the participation of LCN2/24p3R signaling in cell proliferation or apoptotic mechanisms, nor the gonadotropic stimulus regulation of such signaling.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EDLC participated in the conception and design of the study, performed the immunohistochemistry experiments, analysis and interpretation of data, and prepared the manuscript. LMA, LD, AO, MAP and IS collected the biological samples, performed the semi-quantitative reverse transcription polymerase chain reaction and western blot analyses, and analysed and interpreted data. JPM participated in the design of the study, prepared the manuscript and revised the manuscript for its intellectual content. Also, the final version of the manuscript has been read and approved by all authors and each author believes the manuscript represents honest work.

Ethical approval and consent to participate

The experimental protocol was approved by the Research Committees of the National Medical Center and the National Autonomous University of Mexico (México City, México; approval no. UNAM-003-2013).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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