Dyslipidemia alters sperm maturation and capacitation in LXR-null mice

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Abstract

Lipid metabolism disorders (dyslipidemia) are causes of male infertility, but little is known about their impact on male gametes when considering post-testicular maturation events, given that studies concentrate most often on endocrine dysfunctions and testicular consequences. In this study, three-month-old wild-type (*wt*) and Liver-X-Receptors knock out (*Lxrα;β*^{-/-}) males were fed four weeks with a control or a lipid-enriched diet containing 1.25% cholesterol (high cholesterol diet (HCD)). The HCD triggered a dyslipidemia leading to sperm post-testicular alterations and infertility. Sperm lipids were analyzed by LC-MS and those from *Lxrα;β*^{-/-} males fed the HCD showed higher chol/PL and PC/PE ratios compared to *wt*-HCD ($P < 0.05$) and lower oxysterol contents compared to *wt* ($P < 0.05$) or *Lxrα;β*^{-/-} ($P < 0.05$). These modifications impaired membrane-associated events triggering the tyrosine phosphorylation normally occurring during the capacitation process, as shown by phosphotyrosine Western blots. Using flow cytometry, we showed that a smaller subpopulation of spermatozoa from *Lxrα;β*^{-/-}-HCD males could raise their membrane fluidity during capacitation ($P < 0.05$ vs *wt* or *wt*-HCD) as well as their intracellular calcium concentration ($P < 0.05$ vs *Lxrα;β*^{-/-} and $P < 0.001$ vs *wt*). The accumulation of the major sperm calcium efflux pump (PMCA4) was decreased in *Lxrα;β*^{-/-} males fed the HCD ($P < 0.05$ vs *Lxrα;β*^{-/-} and $P < 0.001$ vs *wt*). This study is the first showing an impact of dyslipidemia on post-testicular sperm maturation with consequences on the capacitation signaling cascade. It may lead to the identification of fertility prognostic markers in this pathophysiological situation, which could help clinicians to better understand male infertilities which are thus far classified as idiopathic.

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Introduction

Lipid metabolism disorders (dyslipidemia) have dramatically increased around the world in the last decades, with major effects on health and life expectancy. Among dyslipidemia, hypercholesterolemia affects, for example, more than 30 million Americans who are over the age 20 (13.1% of this population) and present a high blood cholesterol level (>240 mg/dL, (Mozaffarian *et al.* 2015)). Other studies have shown a high prevalence of dyslipidemia in the adult population: again, as an example, 45.3% of French men aged 30–54 years are dyslipidemic without being treated, while only 9.4% of men in this age group are diagnosed and treated (Ferrieres 2008); similarly in Canada, 43% of men over age 20 are also presenting a dyslipidemia (Joffres *et al.* 2013). Even though the negative impact of these diseases on reproductive function is known, very few mechanistic studies on spermatozoa have been published (Whitfield *et al.* 2015).

Liver-X-Receptors α (LXR α – *Nr1h3*) and β (LXR β – *Nr1h2*) are nuclear receptors activated by oxysterols

and act as major regulators of cholesterol homeostasis in various cell types (reviewed by Maqdas *et al.* (2016)). The importance of LXRs in the male reproductive physiology was underlined by the *Lxrα;β*^{-/-} mouse model in which male mice showed infertility starting at five months evolving in sterility at 10 months (Volle *et al.* 2007). This phenotype was composite, with alterations of both testis (Volle *et al.* 2007) and epididymis (Frenoux *et al.* 2004, Ouvrier *et al.* 2009, Whitfield *et al.* 2016). We previously showed that the epididymal phenotype could be triggered in young *Lxrα;β*^{-/-} animals by an isocaloric high cholesterol diet (HCD) even though the testis showed no structural and functional alterations (Ouvrier *et al.* 2011). This study revealed the peculiar susceptibility of the epididymis to cholesterol overload and provided a new model of infertility issued from diet-induced hypercholesterolemia. A link between LXR gene polymorphisms and obesity has previously been described in humans (Solaas *et al.* 2010), suggesting a possible link with male infertility; future studies will be necessary to investigate this point.

In this model, the HCD-induced epididymal alterations appeared to affect male gamete functions, in complete agreement with the roles played by epididymal maturation in the acquisition of sperm-fertilizing ability (Cornwall 2009). It is well known that epididymal maturation extensively modifies the sperm protein, sugar and lipid contents (Dacheux *et al.* 2003). Regarding membranous lipids, sterols and phospholipids are qualitatively and quantitatively modified in order to prepare spermatozoa for the subsequent events of sperm post-testicular maturation (i.e. capacitation and acrosomal reaction). In mice, a decrease in the sperm cholesterol content was observed during epididymal transit (Rejraji *et al.* 2006).

Capacitation was defined in the early 1950s as a set of functional and biochemical events that occur after ejaculation in the female genital tract (Chang 1951, Austin 1952). Capacitation is triggered by plasma membrane cholesterol loss (Travis & Kopf 2002) toward cholesterol acceptors in the female secretions (Visconti *et al.* 1999). Recent studies have described the participation of oxysterol (cholesterol oxidation products) production during the course of capacitation as facilitating sterol efflux to further increase membrane fluidity (Brouwers *et al.* 2011, Boerke *et al.* 2013). Consequently, sperm plasma membrane fluidity is increased, allowing the activation of ion channels. Intracellular pH (pH_i) (Chavez *et al.* 2012) and intracellular calcium concentration ([Ca²⁺]_i) are increased (Lishko *et al.* 2012), leading to the activation of an intracellular signaling cascade via a rise in cAMP intracellular concentration produced by the soluble adenylyl cyclase sAC (Wertheimer *et al.* 2013). The Ser/Thr kinase PKA (protein kinase A) is consequently activated by cAMP resulting in phosphorylation of numerous targets. Tyrosine kinases are activated, which will in turn phosphorylate specific tyrosine residues on sperm proteins (Visconti *et al.* 1995). Ultimately, gametes acquire a hyperactive motility (Nassar *et al.* 1999) and the ability to bind and fertilize an oocyte.

Based on our mouse model of *Lxra*; $\beta^{-/-}$ males fed for four weeks with an HCD, this work investigates the impact of the dyslipidemia-induced epididymal disruption on sperm post-testicular lipid composition and maturation, especially during the *in vitro* capacitation process.

Materials and methods

Animals

Mice (*Lxra*; $\beta^{-/-}$ and wt) were derived from a mixed strain background (C57BL/6:129Sv) and were housed in an animal facility with a controlled environment (22°C, 12 h light/12 h dark). Control mice were fed *ad libitum* with a Global-diet 2016 (Harlan, Gannat, France). Under HCD, three-month-old males were fed for four weeks with a lipid-enriched diet containing 1.25% cholesterol (Safe, Augy, France). Mouse housing and manipulations were approved by the Regional

Ethic Committee in Animal Experimentation (authorization CE 100-12). Mice were killed by cervical dislocation.

Sperm collection

For all experiments, sperm were collected by pressure on *cauda* epididymis in 1.0 mL of non-capacitating WH medium (see composition later) and then counted using a Malassez hemocytometer.

Cholesterol and phospholipids assays

Cholesterol and phospholipids assays were carried out at the MetaToul platform (Toulouse). Lipids of sperm (four million) from wt, wt-HCD, *Lxra*; $\beta^{-/-}$ and *Lxra*; $\beta^{-/-}$ HCD were extracted by the Bligh and Dyer method (Bligh & Dyer 1959) with internal standard. Regarding phospholipids, the calibration range was performed for each compound, and then extracts and range were analyzed by liquid chromatography (LC-ESI-QqQ 6460 Agilent). Sterols were silylated and then analyzed by gas chromatography (GC-MS simple quadrupole).

Oxysterols assay

Total lipids were extracted from a pool of six million spermatozoa according to the method of Folch *et al.* (1957). Oxysterols were quantified by gas chromatography coupled to mass spectrometry according to previously published procedures (Grandgirard *et al.* 2004, Fourgeux *et al.* 2012). After a known quantity of betulin (Sigma-Aldrich) was added as an internal standard, samples were submitted to alkaline hydrolysis in KOH before oxysterols were extracted with dichloromethane. Oxysterols were purified on silica columns with acetone and derivatized into trimethylsilyl ethers by the addition of pyridine and BSTFA. Trimethylsilyl ethers were analyzed on a Shimadzu QP2010 gas chromatograph coupled to a mass spectrometer (Shimadzu, Marne-La-Vallée, France). Samples were injected in a splitless mode at an injection temperature of 290°C on a DB-5MS fused silica capillary column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μ m, Agilent Technologies). Hydrogen was used as the carrier gas (inlet pressure 60 kPa). The flame ionization detector was maintained at 290°C. The initial oven temperature was held at 50°C for one minute, and then increased at a rate of 20°C/min to 250°C and thereafter at 5°C/min to a final temperature of 300°C. Electron impact ionization was used at 70 eV ionization energy. Oxysterols were analyzed in the single ion monitoring mode at *m/z* 456 for 7 α -hydroxycholesterol and 7 β -hydroxycholesterol, at *m/z* 474, *m/z* 366 and *m/z* 356 for α -epoxycholesterol and β -epoxycholesterol, at *m/z* 472 and *m/z* 382 for 7-ketocholesterol and at *m/z* 483 and *m/z* 496 for betulin. Absolute amounts of each oxysterol were determined by interpolation from a standard curve of betulin generated for each experiment.

Capacitation media

The basic medium used throughout these studies for sperm preparation and *in vitro* capacitation was a modified Krebs-

Ringer medium (Whitten's-HEPES buffer (WH) containing 100mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4 , 1.2mM MgSO_4 , 5.5mM glucose, 1mM pyruvic acid, 4.8mM lactic acid, 20mM HEPES, pH 7.4). Capacitation medium 1 contained 5mg/mL fatty-acid-free BSA (Sigma-Aldrich), 2mM CaCl_2 and 20mM NaHCO_3 in WH. Capacitation medium 2 was made with WH supplemented with 1mM 8-Bromo-cAMP (Sigma) and 500 μM IsoButylMethylXanthine (IBMX, Sigma). For BSA range experiments, capacitation medium 1 was used with increasing fatty-acid-free BSA concentrations (0–0.3–1–3–5–10–20–30mg/mL).

Analysis of capacitation by Western blot

Sperm were incubated in capacitation medium 1 or 2 at the final concentration of 15×10^6 cells/mL during 0, 45 or 90min at 37°C, 5% CO_2 . Each time, one million was collected, centrifuged at 500g for five minutes, washed with 300 μL of phosphate buffered saline (PBS) and then resuspended in sample buffer without mercaptoethanol and boiled for five minutes. After a centrifugation at 7000g for five minutes, the supernatant was collected, boiled in the presence of 5% mercaptoethanol (Sigma) for five minutes and then subjected to SDS-PAGE as described later.

Extraction of sperm proteins

Cauda epididymal sperm proteins were extracted in RIPA buffer (50mM Tris, 150mM NaCl, 12mM sodium deoxycholate, 3mM sodium dodecyl sulfate, 1% Igepal CA-630, Sigma) supplemented with protease inhibitors (Complete mini, Roche) and phosphatase inhibitors (Halt phosphatase inhibitor cocktail, ThermoScientific) and subjected to 30min of sonication (30s/30s) at 4°C. Samples were then centrifuged at 14,000g for 10min at 4°C. Supernatants were collected and the proteins were dosed by the bicinchoninic acid method. For electrophoresis, 25 μg of proteins was diluted in Laemmli sample buffer and incubated for five minutes at 100°C.

Extraction of tissue proteins

Testis, *caput* and *cauda* epididymal tissues were homogenized in high salt buffer (25mM HEPES, 0.4M NaCl, 1.5mM MgCl_2 , 0.2mM EDTA, 1% Igepal CA-630, Sigma) supplemented with protease and phosphatase inhibitors. Lysates were centrifuged at 4°C for 10min at 13,000g. Supernatants were collected and the proteins were dosed by the Bradford method. For electrophoresis, 25 μg of proteins was diluted in Laemmli sample buffer and incubated for five minutes at 100°C.

Collection of epididymosomes

Cauda epididymides from six mice were collected in 500 μL of WH. Sperm cells were retrieved in WH by first stitching tissues using a needle, and sperm suspensions were collected. Then, tissues were minced in 500 μL of WH to release epididymal fluid. Suspensions of tissues and sperm were centrifuged separately at 500g for five minutes at 4°C. The supernatants were further clarified by centrifugation (16,000g, 20min

at 4°C) and pooled, representing the epididymal luminal fluid (ELF). Using an Optima L-80 XP centrifuge with SW41 rotor (Beckman Coulter, Roissy CDG, France), the ELF was subjected to ultracentrifugation at 120,000g for two hours at 4°C. The pellet, containing the epididymosomes, was taken up in 25 μL RIPA buffer, and the proteins were extracted by 30min of sonication as described earlier. Supernatants were collected and the proteins were dosed by the bicinchoninic acid method. For electrophoresis, 25 μg of proteins were diluted in Laemmli sample buffer and incubated for five minutes at 100°C.

Immunoprecipitation of phosphotyrosine-containing proteins

Cauda epididymal sperm were collected as described earlier, and 80million sperm cells were incubated in capacitation medium 1. At 0 and 90min of incubation, sperm cells (40million) were washed in PBS, and proteins were extracted in RIPA with protease and phosphatase inhibitors. After sonication as described earlier, lysates were centrifuged at 4°C for 10min at 15,000g. Supernatants were incubated 30min at 4°C with Protein A/G magnetic beads (ThermoScientific) for preclearing. Then, 20 μL of supernatants was mixed with 5 μL of Laemmli 5 $\times$ and boiled for five minutes (input). The remainder of the supernatants was subjected to immunoprecipitation by incubation with 20 μL of magnetic beads conjugated with the anti-phosphotyrosine antibody (16-282, Merck Millipore), overnight at 4°C. Then, magnetic beads were washed thrice with 300 μL of PBS and boiled for five minutes in 20 μL of Laemmli 2 $\times$ before SDS-PAGE as described later.

SDS-PAGE and Western blot analysis

Protein samples were subjected to denaturing SDS-PAGE and transferred on nitrocellulose membrane (Hybond ECL, Amersham Biosciences). Blots were blocked with Tris Buffered Saline (50mM Tris, 150mM NaCl) containing 0.1% v/v Tween 20 and 10% w/v low-fat dried milk for anti-phosphotyrosine (05-321, clone 4G10, Merck Millipore), α -Tubulin (T6074, Sigma) and β -Actin (A2066, Sigma) antibodies, 5% w/v BSA for VDAC-2 antibody (ab37985, ABCAM) or 5% w/v low-fat dried milk and 3% casein for PMCA4 antibody (sc-22080, Santa Cruz Biotechnology). Membranes were probed overnight at 4°C with anti-phosphotyrosine (1/1000), α -Tubulin (1/10,000), β -Actin (1/1000), VDAC2 (1/250) and PMCA4 (1/500) antibodies in corresponding blocking solutions. After washing, membranes were incubated with the following secondary antibodies: anti-mouse horseradish peroxidase-conjugated (HRP) (BI 2413C, 1/1000 for anti-phosphotyrosine and α -Tubulin; Abliance, Compiègne, France), anti-rabbit HRP (BI 2407, 1/1000 for β -Actin, Abliance) and anti-goat HRP (BI 2403, 1/1000 for VDAC2, Abliance). For PMCA4, anti-goat Biotin-SP conjugated (1/5000, 705-066-147, Jackson Immunoresearch) followed by streptavidine HRP (1/5000, 016-030-084, Invitrogen) were used. Detection was performed using the Immobilon Western Chemiluminescent HRP substrate (Millipore) on MF-ChemiBIS 3.2 camera (DNR Bioimaging systems, Jerusalem, Israel), and densitometric analyses were carried out using Multi Gauge V3.2 software.

Table 1 Sequences of the primers used in quantitative RT-PCR (Reverse Transcription-Polymerase Chain Reaction) experiments.

Gene	NCBI reference #	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)
36b4	NM_007475.5	gtc act tgt cca gct cag aa	tca atg gtg cct ctg gag at	98
Pmca4	NM_001167949.2	ctg caa ggc aaa ctc aca cg	act cag gaa gcc att ctc gg	139
Vdac2	NM_011695.2	aag acg aag tca tgc agc gg	cgg tgt tcc act tct ctg tga	139

Evaluation of membrane fluidity using flow cytometry

Sperm (15×10^6 cells/mL) were incubated in capacitation medium 1 during 0, 45 and 90 min. Each time, after washing in WH, $2.7 \mu\text{M}$ of merocyanine 540 (marker of membrane fluidity, Molecular Probes) and $25 \mu\text{M}$ of YO-PRO-1 (a marker of viability, Molecular Probes) were added and 0.5×10^6 sperm cells were acquired in LSR II (BD Biosciences, San Jose, CA, USA) flow cytometer, and the results were analyzed using FACSDIVA 6.01 software.

Determination of calcium flux using flow cytometry

Cauda epididymal sperm cells (10×10^6 cells/mL) were loaded with $5.0 \mu\text{M}$ of Fluo4-AM probe (dissolved in 20% Pluronic F127 to facilitate cell loading) for one hour at room temperature in the dark (Molecular Probes) in WH. After washing in WH, sperm were incubated in capacitation medium 1 for 0, 45 and 90 min. Before each assay, $1.5 \mu\text{g/mL}$ of propidium iodide (a marker of cell viability, Sigma) was added and 0.5×10^6 sperm cells were acquired on an LSR II (BD Biosciences) flow cytometer, and the results were analyzed using FACSDIVA 6.01 software.

Immunocytology

Sperm (5×10^6 cells/mL) were fixed in paraformaldehyde 2% in PBS for 15 min at 4°C . After two washes in PBS, sperm were spread on glass slides. After drying at room temperature, sperm were permeabilized with PBS-triton 0.2% for 20 min and blocked one hour with PBS-triton 0.2%, NGS 1.5% (normal goat serum, Vector Laboratories, Burlingame, CA, USA). Slides were then probed with anti-phosphotyrosine 4G10 antibody (1/100, Merck Millipore) overnight at 4°C . The secondary anti-mouse Alexa Fluor 555-conjugated antibody (1/100, A21425, Life Technologies) was incubated one hour at room temperature, and sperm nuclei were stained with Hoechst 33342 ($1 \mu\text{g/mL}$, Invitrogen) and mounted on PBS/glycerol. The percentages of unlabeled, tail, head and head+tail stains were evaluated by counting at least 200 cells per slide.

Quantification of mRNA by Real-Time Quantitative RT-PCR

Total mRNAs from testis, *caput* and *cauda* epididymides were isolated using the NucleoSpin RNA II column kit (Macherey-Nagel, Düren, Germany), according to manufacturer's instructions. RNA ($1 \mu\text{g}$) was reverse-transcribed, and quantitative PCR was performed as previously described (Whitfield et al. 2016). The relative accumulation level of each mRNA was normalized using 36b4 as a standard and analyzed with the $\Delta\Delta\text{Ct}$ method. Primer sequences are listed in Table 1.

Results

Dyslipidemia affects sperm lipid composition

To test whether the diet-induced epididymal phenotype affects the sperm lipid content, we assayed the cholesterol and phospholipid composition in *cauda* epididymal sperm of four-month-old *wt* or *Lxr α ; β ^{-/-}* male mice fed a control or HCD diet. No significant difference in cholesterol or total phospholipids was apparent between groups (Fig. 1A). However, the cholesterol/phospholipid (Chol/PL) ratio and the phosphatidylethanolamine/phosphatidylcholine (PE/PC) ratio were significantly increased in sperm from *Lxr α ; β ^{-/-}*-HCD mice ($P < 0.05$ compared to *wt*-HCD, Fig. 1A). As recent articles have described the presence of oxysterols in sperm (Brouwers et al. 2011, Boerke et al. 2013) thought to promote membrane fluidity by causing lateral membrane expansion (Olkkonen & Hynynen 2009), we assessed the amount of oxysterols in *cauda* sperm cells by mass spectrometry. Total oxysterols were significantly decreased in *Lxr α ; β ^{-/-}*-HCD compared to *wt* and *Lxr α ; β ^{-/-}*, mainly due to a decrease in 7α -hydroxycholesterol, 7β -hydroxycholesterol, 7-ketocholesterol and β -epoxycholesterol ($P < 0.05$ compared to *wt* and *Lxr α ; β ^{-/-}*, Fig. 1B). Overall, these data suggest that dyslipidemia disrupts the epididymal function and impairs sperm lipid composition in *Lxr α ; β ^{-/-}* males fed with the HCD.

Dyslipidemia disrupts membrane-related capacitation events

Cholesterol efflux from the sperm plasma membrane facilitated by acceptors such as albumin present in the secretions of the female genital tract triggers sperm capacitation. This can be mimicked *in vitro* by adding BSA in the capacitation medium (Fig. 2A-1), and the efficiency of capacitation can be measured by quantifying tyrosine-phosphorylated proteins by Western blot, as it is a final marker of the process.

As shown in Fig. 2A1, the HCD had no effect on capacitation efficiency in *wt* animals, during a kinetic study between 0, 45 and 90 min of incubation. *Lxr α ; β ^{-/-}* males showed a rather altered tyrosine phosphorylation profile that was slightly aggravated by the HCD. Since signaling pathway following sperm plasma membrane cholesterol efflux involves sAC-mediated cAMP production, which in turns activates PKA resulting *in fine* in tyrosine phosphorylation events, we have used 8-bromo-cAMP (a cell-permeant analog of cAMP) and IBMX (an

Figure 1 Influence of HCD on sperm lipid composition. (A) Cholesterol and phospholipids were assayed and membrane-fluidity-related ratios were calculated on sperm from control diet- and HCD-fed four-month-old wild-type (wt) and *Lxra;β*^{-/-} mice. Horizontal bars represent means $\pm$ S.E.M., $n=4$. (B) Total and different classes of oxysterols were assayed from sperm of control and HCD-fed four-month-old wild-type (wt) and *Lxra;β*^{-/-} mice. Horizontal bars represent means $\pm$ S.E.M., $n=6$ wt, 4 wt-HCD, 5 *Lxra;β*^{-/-}, 3 *Lxra;β*^{-/-} HCD. * $P<0.05$ between the compared groups (Mann–Whitney test). Chol, cholesterol; PL, phospholipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

inhibitor of the phosphodiesterases responsible for cAMP degradation) to produce an increase in intracellular cAMP. In doing so, PKA is activated independently of the physiological membrane events (cholesterol efflux) normally triggering the capacitation process. Under these conditions (Fig. 2A–2), tyrosine phosphorylation of sperm proteins during capacitation was similar between groups. This result strongly suggests that the dysregulations observed with BSA as a capacitation inducer are due to membrane defaults, upstream of cAMP production. This hypothesis was further tested by incubating sperm in capacitation media containing increasing amounts of BSA (Fig. 2B). For wt sperm, fed with control diet or HCD, the maximum efficiency was reached when low concentrations of BSA were present (left and right upper panels). Maximum efficiency in tyrosine phosphorylation was reached with higher BSA concentrations for *Lxra;β*^{-/-} males (lower left panel) and *Lxra;β*^{-/-}-HCD males (lower right panel). These results are in accordance with changes in membrane dynamics limiting cholesterol efflux to extracellular acceptors, and thus altering capacitation efficiency.

To determine if the decrease in tyrosine phosphorylation was due to a decrease in the number of capacitating sperm cells or to a decrease in the overall phosphorylation intensity, the percentage of sperm cells stained by the anti-phosphotyrosine antibody was calculated (Fig. 3A). It clearly appeared that significantly fewer spermatozoa were stained in the *Lxra;β*^{-/-} males compared to the wt group at any time of capacitation (a). These results also showed that sperm from *Lxra;β*^{-/-}-HCD mice were significantly less stained than the ones from *Lxra;β*^{-/-} males (c), here again in accordance with the Western blot data. At t0, fewer wt-HCD spermatozoa were stained compared to wt, but this difference was

not maintained during the course of capacitation. The defects in the capacitation-associated signaling pathway were probably located on the whole sperm cell surface as the percentages of sperm cells stained on the head or the tail were lower in the *Lxra;β*^{-/-} males fed or not with the HCD compared to the wt groups at any time of capacitation (Fig. 3B).

Dyslipidemia alters sperm membrane fluidity during capacitation

Sperm plasma membrane fluidity was evaluated using a direct method with merocyanine-540 (M-540) that has the ability to target membrane fluid domains. M-540 detection was coupled with YO-PRO-1 (detecting live cells), and flow cytometry was used to measure the relative proportion of live sperm undergoing membrane fluidification during the capacitation process. In Fig. 4A, living spermatozoa (in green in the Q4 quartile) appeared as separated into two subpopulations (P2 and P3): P2 showing low membrane fluidity and P3 showing high membrane fluidity. During the course of capacitation, the relative proportion of the P3 subpopulation increased, revealing an increase in the percentage of sperm cells showing high fluidity (lower panel of Fig. 4A). When comparing the percentages of sperm cells in the P3 subpopulation between groups, there was no significant difference at t0 (Fig. 4B, left panel). At that time, the heterogeneity was high, and a tendency to a lower membrane fluidity was visible for *Lxra;β*^{-/-} males fed the normal diet or the HCD. During capacitation, at t45 and t90, significantly fewer spermatozoa reached a state of high membrane fluidity for the *Lxra;β*^{-/-} males fed with the HCD compared to wt and wt-HCD spermatozoa (Fig. 4B, middle and right

Figure 2 Capacitation efficiency determined with anti-phosphotyrosine Western blot. (A) Sperm from *wt* and *Lxra;β^{-/-}* mice fed with control diet or HCD were incubated 0, 45 or 90 min in classical capacitation medium 1 or in medium 2 supplemented with 8-Bromo-cAMP and IBMX. Protein extracts were analyzed with anti-phosphotyrosine antibody. This experiment was performed four times with similar results. Alpha-tubulin was used as a control of the protein amount. (B) Sperm from *wt* and *Lxra;β^{-/-}* mice fed with control diet or HCD were incubated 90 min in capacitation medium 1 containing increasing concentrations of BSA (0, 0.3, 1, 3, 5, 10, 20, 30 mg/mL). Protein extracts were analyzed with anti-phosphotyrosine antibody. Each Western blot for each group is representative of four experiments. Alpha-tubulin was presented under each blot as a control of protein amount.

Figure 3 Evaluation of the percentage of tyrosine-phosphorylated sperm during capacitation. Sperm from control diet and HCD-fed wt and *Lxra*;β^{-/-} mice were incubated during 0, 45 or 90 min in capacitation medium 1 prior tyrosine phosphorylation immunostaining. (A) After counting, the percentage of tyrosine-phosphorylated sperm was evaluated. The points are means ± S.E.M. (B) Representation of subcellular staining distribution (not stained, tail stained, head stained or both) in percentage for each group at each time of capacitation. At least 200 sperm were counted per experiment, $n=4$, ^a $P<0.05$ vs wt, ^b $P<0.05$ vs wt-HCD, ^c $P<0.05$ vs *Lxra*;β^{-/-} (Mann-Whitney test).

Figure 4 Evaluation of membrane fluidity using flow cytometry. Sperm from wt and *Lxra* β ^{-/-} mice fed with control diet or HCD were incubated 0, 45 or 90 min in capacitation medium 1. YO-PRO-1 and merocyanine-540 were added to determine sperm viability and membrane fluidity, respectively, and 0.5×10^6 cells were acquired on an LSR II flow cytometer. (A) The upper panel shows cytograms with a green population representing live sperm stained with merocyanine-540 probe. The underlying histograms show the cell number of this green subpopulation depending on the intensity of merocyanine-540 fluorescence. Two subpopulations are visible: P2 with low membrane fluidity and P3 with high membrane fluidity. Horizontal bars represent means. (B) Scatter dot plots represent the percentage of live sperm in P3 subpopulation (high membrane fluidity) during capacitation. (C) Evolution of median fluorescence in merocyanine-540 in the green subpopulation during capacitation. The points are means $\pm$ S.E.M. * $P < 0.05$ between compared groups, ^a $P < 0.05$ vs wt, ^b $P < 0.01$ vs wt-HCD (Mann-Whitney test). $n = 8$ wt, 9 wt-HCD, 9 *Lxra* β ^{-/-}, 8 *Lxra* β ^{-/-} HCD. M-540, merocyanine-540.

panels). There was also a similar tendency for $Lxr\alpha;\beta^{-/-}$ males fed with the control diet (compared to *wt*) at these two times of capacitation.

The median of fluorescence intensity measured on the Q4 subpopulation is related to the overall sperm membrane fluidity as it is dependent on the total amount of M-540 inserted in their membrane. When plotting this median of fluorescence to compare the groups, no significant difference was seen at t0. During the course of capacitation, it was clear that $Lxr\alpha;\beta^{-/-}$ males fed with the control diet or the HCD had a significantly lower overall fluidity than the *wt* spermatozoa (Fig. 4C).

Overall, these results showed that fewer spermatozoa from $Lxr\alpha;\beta^{-/-}$ males fed with the HCD had the ability to engage into capacitation as measured by the increase in membrane fluidity, and that their overall fluidity was lower than for the *wt* spermatozoa.

Dyslipidemia alters sperm calcium fluxes during capacitation

Calcium influx during capacitation is highly regulated and involved in the control of the signaling pathway triggered by membrane events. Alterations of the membrane dynamics may thus have impacts on these calcium fluxes. We used flow cytometry with Fluo4-AM to measure $[Ca^{2+}]_i$ in capacitating spermatozoa from our four groups (Fig. 5). As seen for membrane fluidity in Fig. 4, living sperm in the Q4 quartile (Fig. 5A in green) were separated into two subpopulations of low (P2) and high (P3) $[Ca^{2+}]_i$. During the course of capacitation, the percentage of sperm cells in the P3 subpopulation normally increases as seen in the lower panel of Fig. 5A. At t0, the differences between groups were minimal and $[Ca^{2+}]_i$ was low. There was a significant difference between *wt*-HCD and $Lxr\alpha;\beta^{-/-}$ -HCD (Fig. 5B left panel, $P < 0.05$). During capacitation, the rise in $[Ca^{2+}]_i$ was clearly visible between t0 and t45 for all the groups (Fig. 5B, middle panel). Fewer spermatozoa from the $Lxr\alpha;\beta^{-/-}$ males showed a high $[Ca^{2+}]_i$, with an effect of the diet at t90 as the difference was significant between $Lxr\alpha;\beta^{-/-}$ and $Lxr\alpha;\beta^{-/-}$ -HCD males (Fig. 5B, right panel, $P < 0.05$).

Here also, the median of fluorescence in the Q4 subpopulation was used to evaluate the relative $[Ca^{2+}]_i$ between groups. At t0, even though no significant difference appeared between groups, sperm from the $Lxr\alpha;\beta^{-/-}$ -HCD males had a higher $[Ca^{2+}]_i$ than those from *wt* males, and this tendency was inverted at t90 (Fig. 5C, left panel). The ratio of the median fluorescence between t90 and t0 was calculated, and indicated the capacity of the sperm cells to elevate their $[Ca^{2+}]_i$ during the course of capacitation. The difference was significant between $Lxr\alpha;\beta^{-/-}$ -HCD and each of the other groups (Fig. 5C, right panel).

Overall, these results showed that fewer spermatozoa from $Lxr\alpha;\beta^{-/-}$ males fed with the HCD had the ability

to engage in capacitation, as measured by the increase in $[Ca^{2+}]_i$, and that their overall $[Ca^{2+}]_i$ was lower than for the other groups at the end of capacitation. These results are in accordance with those from membrane fluidity measurements (Fig. 4). Indeed, there is a strong correlation between the subpopulations of high membrane fluidity and high intracellular calcium concentration at t90 (Fig. 6A, $r = 0.5972$, $P < 0.0002$). Increasing the BSA concentration in the capacitation medium from 5 to 20 mg/mL provoked a slight non-significant increase in the percentage of spermatozoa showing a high $[Ca^{2+}]_i$ after capacitation, specifically in $Lxr\alpha;\beta^{-/-}$ males (Fig. 6B). This may explain the impact of BSA concentration on tyrosine phosphorylation shown in Fig. 2.

Sperm calcium dysregulation comes from dyslipidemia-induced epididymal sperm maturation defect

Calcium fluxes during capacitation are regulated by the activity of several proteins, including the major calcium efflux pump PMCA4 (plasma membrane calcium ATPase 4) and VDAC2 (voltage-dependent anion channel 2). VDAC2 was previously demonstrated as tyrosine-phosphorylated during capacitation (Arcelay et al. 2008), while PMCA4 is known to be acquired during epididymal transit (Patel et al. 2013). These characteristics prompted us to evaluate their behavior in the $Lxr\alpha;\beta^{-/-}$ -observed dysregulations of $[Ca^{2+}]_i$. We therefore first investigated the distribution of these proteins on *cauda* epididymal sperm from our four groups by Western blot. PMCA4 was shown to be significantly decreased in sperm from $Lxr\alpha;\beta^{-/-}$ males fed with the HCD (compared to each of the other groups), whereas VDAC2 was stable among the groups (Fig. 7A). As PMCA4 has been shown to be acquired via epididymosomes during epididymal maturation, these vesicles were isolated from the *cauda* epididymis of *wt* and $Lxr\alpha;\beta^{-/-}$ -HCD males. The quantities of PMCA4 and VDAC2 were then measured by Western blot (Fig. 7B). Both PMCA4 and VDAC2 were less abundant on epididymosomes purified from $Lxr\alpha;\beta^{-/-}$ -HCD males compared to *wt* males, even though these differences were not statistically significant due to variations between the different pools used for this study ($P = 0.3$ for PMCA4 and 0.0571 for VDAC2).

Tyrosine phosphorylation of VDAC2 and PMCA4 (although it is not yet known whether PMCA4 is Tyr-phosphorylated during capacitation) was evaluated, and tyrosine-phosphorylated proteins were immunoprecipitated from sperm of *wt* and $Lxr\alpha;\beta^{-/-}$ -HCD males, at t0 and t90 of the capacitation process. VDAC2 and PMCA4 were measured by Western blot on the pool of immunoprecipitated proteins in order to evaluate the course of their tyrosine phosphorylation during capacitation. As previously described, VDAC2 was tyrosine-phosphorylated during capacitation under our two conditions (Fig. 8A). However, the level of

Figure 5 Determination of calcium fluxes during capacitation. Sperm from wt and $Lxra;\beta^{-/-}$ mice fed with control diet or HCD were incubated one hour with Fluo4-AM (to load sperm). Fluo4-AM emits fluorescence depending on intracellular calcium concentration as the probe is blocked in cells after hydrolysis of the ester group by cell esterase. Then, sperm were incubated 0, 45 or 90 min in capacitation medium 1, Pi was added to determine sperm viability and 0.5×10^6 cells were acquired on an LSR II BD flow cytometer. (A) The upper panel shows cytograms with a green subpopulation representing live sperm that integrated Fluo4-AM probe. The underlying histograms show the cell number of this green subpopulation depending on the intensity of Fluo4-AM fluorescence. Two subpopulations are visible: P2 with low intracellular calcium concentration ($[Ca^{2+}]_i$) and P3 with high $[Ca^{2+}]_i$. (B) Scatter dot plots represent the percentage of live sperm in P3 subpopulation (high intracellular calcium concentration) during capacitation. Horizontal bars represent mean. (C) Evolution of the median fluorescence of Fluo4-AM in green subpopulation during capacitation (left panel), and median fluorescence ratio of t90 to t0 indicating the increase in $[Ca^{2+}]_i$ during capacitation (right panel). The values are means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between compared groups (Mann–Whitney test). $n = 8$ wt, 9 wt-HCD, 9 $Lxra;\beta^{-/-}$, 8 $Lxra;\beta^{-/-}$ HCD. Pi, propidium iodide.

Figure 6 Correlation between high membrane fluidity and high intracellular calcium concentration sperm subpopulations at the end of capacitation (t90) and impact of BSA concentration on calcium fluxes. (A) Percentages of spermatozoa with high membrane fluidity were plotted against percentages of spermatozoa with high $[Ca^{2+}]_i$ at $t=90$ min of capacitation. Spearman correlation was used to examine the association between these two subpopulations. $r=0.5972$, $P<0.0002$ (***), significance was determined at a confidence level of 95%. (B) The calcium fluxes were determined by flow cytometry as described in Fig. 4. The graphs represent the percentage of live sperm with high intracellular calcium concentration during capacitation in a medium containing 5 or 20 mg/mL of BSA. The values are means $\pm$ S.E.M. ($n=4$ for each point).

VDAC2 tyrosine phosphorylation was higher at t0 in spermatozoa from $Lx\alpha;\beta^{-/-}$ -HCD males compared to wt (Fig. 8A). When considering the fold of VDAC2 tyrosine phosphorylation during the course of capacitation, it was close to 2 for wt sperm, whereas it was slightly above 1 for $Lx\alpha;\beta^{-/-}$ -HCD males (Fig. 8B). PMCA4 also showed tyrosine phosphorylation at t0, but capacitation had no

Figure 7 PMCA4 and VDAC2 protein levels in cauda sperm and epididymosomes. (A) Relative PMCA4 and VDAC2 protein levels in cauda sperm from wt and $Lx\alpha;\beta^{-/-}$ mice fed with control or HCD diet. Alpha-tubulin was used as a control of protein amount for quantification. Bar graphs display means $\pm$ S.E.M., $n=10$ wt, 13 wt-HCD, 11 $Lx\alpha;\beta^{-/-}$, 16 $Lx\alpha;\beta^{-/-}$ HCD. (B) Relative PMCA4 and VDAC2 protein levels in cauda epididymosomes from wt and $Lx\alpha;\beta^{-/-}$ HCD (each point represents epididymosomes from six mice). Beta-actin was used as a control of protein amount for quantification. In scatter dot plots, horizontal bars represent means $\pm$ S.E.M., $n=4$. * $P<0.05$, *** $P<0.001$ between compared groups (Mann-Whitney test).

effect on its level (Fig. 8C and D). For these two proteins, a certain heterogeneity appeared between individuals.

Overall, these data showed that spermatozoa from $Lx\alpha;\beta^{-/-}$ -HCD males had lower levels of PMCA4 than wt spermatozoa, and that the tyrosine phosphorylation of VDAC2 was altered at t0. The importance of these alterations in the dysregulations of $[Ca^{2+}]_i$ will be discussed.

Discussion

Fat-rich regimens are mainly responsible for several serious and widespread illnesses such as obesity, diabetes and cardiovascular diseases. Such regimens are also known to impact reproductive capacities both in males and females (Seli et al. 2014, Katib 2015). In males, the focus has mainly been put on lipid-driven endocrine

Figure 8 Analysis of PMCA4 and VDAC2 tyrosine phosphorylation during capacitation. Sperm from wt and *Lxra;β^{-/-}* HCD mice were incubated for 0 or 90 min in capacitation medium 1. Each time, total sperm proteins were extracted (40 million) and subjected to tyrosine-phosphorylated immunoprecipitation (IP). Input (representing a total protein amount before immunoprecipitation) and IP samples were subjected to SDS-PAGE and Western blotting was performed against VDAC2 (A) and PMCA4 (C). Scatter dot plots represent IP normalization with input. The increase in tyrosine phosphorylation for VDAC2 (B) and PMCA4 (D) during capacitation was calculated. In scatter dot plots, horizontal bars represent means ± S.E.M. (PMCA4 $n=6$ wt, 5 *Lxra;β^{-/-}* HCD and VDAC2 $n=4$ wt, 6 *Lxra;β^{-/-}* HCD).

dysregulations of spermatogenesis. However, recent data pinpointed the epididymis and the post-testicular sperm maturation as an additional target of metabolic disorders, with the capacity to modify maturing sperm, ultimately leading to the eventual transmission of the disorders to the offspring (Chen *et al.* 2016, Sharma *et al.* 2016). Our data are totally in accordance with the observation that structure and functions of accessory organs of the male genital tract are impaired by diet-induced lipid disorders. We show that in our model of *Lxra;β^{-/-}*-HCD-fed mice, dyslipidemia alters the epididymal function, sperm lipid composition and dynamics, as well as protein acquisition via epididymosomes, with negative consequences on sperm capacitation.

Dyslipidemia alters sperm lipid composition

In this model of *Lxra;β^{-/-}*-HCD-fed mice, the high level of susceptibility of epididymis to dietary cholesterol overload is highlighted. We had previously shown that cholesterol overload resulted in serious epididymal phenotype, characterized by intra-epithelial lipid accumulation, epithelium degeneration and the creation of an inflammatory environment, leading to name this phenotype 'epididymosclerosis' (Ouvrier *et al.* 2011). Here, we observed that epididymal maturation was disrupted with an impact on both sperm lipids and proteins. Lipid changes in *Lxra;β^{-/-}*-HCD sperm were subtle but sufficient to increase the Chol/PL and PE/PC ratios, translating into membrane stiffening. We could also observe an overall decrease in the oxysterol content in these spermatozoa. This is in favor of membrane

stiffening, as oxidized sterols are known to promote membrane fluidity by causing lateral membrane expansion (Olkonen & Hynynen 2009). Oxysterols have recently been described in bovine (Brouwers *et al.* 2011), porcine and mouse sperm (Boerke *et al.* 2013). They were shown to play a role in the capacitation process and to move more freely out of the sperm membrane. They bind to acceptor proteins such as albumin due to their higher hydrophilic characteristics as compared to cholesterol (Aitken 2011). Taken together, these data indicate that the dyslipidemia-induced increase in the Chol/PL and PE/PC ratios associated with the reduction of oxysterols in *Lxra;β^{-/-}*-HCD sperm may result in a decrease in membrane fluidity limiting the sterol efflux that triggers capacitation. Our hypothesis is supported by the observation that increasing the availability of the extracellular cholesterol acceptor by rising BSA concentration in the capacitation medium partially restored the efficiency of capacitation, as measured by tyrosine phosphorylation.

Sperm lipid composition changes impair membrane dynamics and capacitation signaling cascade

Flow cytometry data confirmed that membrane fluidity was impaired in sperm cells from *Lxra;β^{-/-}*-males fed the normal diet or the HCD. At t0, the median of fluorescence indicated a tendency to an increase in membrane rigidity in these spermatozoa, in accordance with the calculated chol/PL and PE/PC ratios. The difference was not significant probably because merocyanine-540 is not sensitive enough to slight fluidity variations, but

this probe revealed significant differences during the course of capacitation. A significantly lower percentage of $Lx\alpha;\beta^{-/-}$ -HCD living sperm cells (dead sperm being excluded) were able to develop a high membrane fluidity during capacitation compared to *wt*. These data attest that spermatozoa in this lipid-mediated abnormal context (i.e. the $Lx\alpha;\beta^{-/-}$ -HCD) have difficulty bringing their plasma membrane to an optimal state of fluidity.

Mammalian spermatozoa are very peculiar cells as their genetic material is silent, due to its very high degree of compaction, enabling its protection during the journey toward the fertilization site. Molecular events occurring during post-testicular maturation are consequently highly dependent on their membrane composition and dynamics. We demonstrated that the major problem for the $Lx\alpha;\beta^{-/-}$ -HCD spermatozoa to achieve capacitation indeed resides at the plasma membrane level since the intracellular signaling events downstream of sAC and upstream of tyrosine kinases were perfectly normal. This was evidenced by the fact that a totally standard tyrosine phosphorylation profile could be obtained *in vitro* in all situations tested when using a classical (8-Bromo-cAMP+IBMX) capacitation medium known to provoke tyrosine phosphorylation in the absence of BSA (Visconti *et al.* 1995).

Calcium fluxes have been described as essential for the regulation of capacitation (Lishko *et al.* 2012). Calcium is involved in the signaling cascade regulating capacitation by participating in the sAC activation (Jaiswal & Conti 2003), in the acquisition of the sperm hyperactivation movement (Navarrete *et al.* 2015) and in the triggering of the acrosome reaction (Navarrete *et al.* 2015). Sperm $[Ca^{2+}]_i$ is finely tuned by a set of ion channels (Lishko *et al.* 2012) because calcium can have a dual role as both an activator and an inhibitor of capacitation (Navarrete *et al.* 2015). During capacitation, fewer $Lx\alpha;\beta^{-/-}$ -HCD sperm were able to acquire a high $[Ca^{2+}]_i$, showing dysregulations in capacitation-associated calcium influx. The observed dysregulations of $[Ca^{2+}]_i$ were more subtle since basal $[Ca^{2+}]_i$ was perturbed in $Lx\alpha;\beta^{-/-}$ with a tendency to be too elevated, and the calculated ratio of $[Ca^{2+}]_i$ increase during capacitation was significantly impaired only in $Lx\alpha;\beta^{-/-}$ -HCD sperm. The most studied calcium channels in relation to capacitation and hyperactivation are the CatSper (cation channels of sperm) channels, which are activated by the rise in intracellular pH during capacitation (Kirichok *et al.* 2006). These channels have been proven as essential for male fertility based on the fact that male mice lacking any of the CatSper1–4 or δ genes are infertile, due to a lack of hyperactivated motility and capacitation defects (Quill *et al.* 2003, Chung *et al.* 2011). However, CatSper1-null spermatozoa show a spontaneous and dramatic increase in tyrosine phosphorylation, which is further enhanced upon capacitation (Chung *et al.* 2014). This is not in accordance with our phenotype in which

hypo-phosphorylation was described. In addition, to our knowledge, Catsper channels have never been described as tyrosine-phosphorylated during capacitation.

Dyslipidemia is associated with a decrease in sperm-PMCA4 accumulation

The regulation of sperm $[Ca^{2+}]_i$ depends on several important ion channels and efflux pumps, a major one being the calcium pump PMCA4. PMCA4 is a family of enzymes that extrude calcium from the cytosol across the plasma membrane of eukaryotic cells. The tissue-specific expression pattern of different isoforms and splice variants of the pump in mammalian tissues strongly suggest a specific physiological function (Strehler & Zacharias 2001). PMCA4-deficient mice have revealed the importance of this isoform in male fertility as males are infertile due to impaired sperm motility, associated with $[Ca^{2+}]_i$ that is two times higher than in wild-type sperm (Okunade *et al.* 2004). The decrease in PMCA4 protein accumulation we observed in our model could explain the tendency for the calcium concentration to increase at t0 of capacitation. PMCA4 is partially acquired by spermatozoa during spermatogenesis in the testes, and is also transferred to sperm during epididymal transit via epididymosomes (Patel *et al.* 2013). In mouse sperm, PMCA4 was localized in lipid rafts over the acrosome and the proximal principal piece (Aravindan *et al.* 2012), and it can be delivered in these regions by extracellular vesicles (oviductosomes) by a fusogenic mechanism (Al-Dossary *et al.* 2015). Our data showed that the decrease in the amount of sperm PMCA4 in $Lx\alpha;\beta^{-/-}$ -HCD sperm was due to a lower epididymosome content and thus probably also due to a default in its acquisition by maturing sperm. The inter-individual heterogeneity observed in the amount of sperm PMCA4 (and VDAC2) may be related to different storage times of caudal spermatozoa, allowing the transfer of higher amounts of proteins for those residing for a longer period. This is supported by the fact that protein accumulation in testes or *caput* epididymis is not different among our four groups (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The difference observed for the *cauda* epididymis is in accordance with this hypothesis. The cause of PMCA4 decrease in spermatozoa remains elusive as it could be related to either a decrease in epididymosomes production, to an alteration of the PMCA4 transfer to spermatozoa, or both. However, it seems that it is not due to a decrease in the expression of the *Pmca4* gene as shown by qPCR data presented in Supplementary Fig. 2, where the expression level was similar between groups in all the tissues explored. A hypothesis could be that dyslipidemia-induced epididymal alterations may modify the lipid composition and thus the membrane structure of epididymosomes. Indeed, these vesicles are characterized by a high cholesterol/phospholipid

ratio and a sphingomyelin enrichment (Rejraji *et al.* 2006). These two characteristics are signatures of lipid raft microdomains, and it was previously shown that PMCA pumps were concentrated in lipid rafts (Fujimoto 1993). As it has also been shown that, during bovine epididymal maturation, proteins could be specifically transferred from rafts and non-rafts domains of epididymosomes to the corresponding sperm domains (Girouard *et al.* 2009), it would be interesting to further investigate epididymosome composition and structure in our model.

A possible link between PMCA4 function and its tyrosine phosphorylation was shown in sensory neurons in which PMCA4 was under tonic inhibition by a member of the Src family of tyrosine kinase (SFKs, (Ghosh *et al.* 2011)). SFKs did not appear to directly phosphorylate PMCA4 but instead activated focal adhesion kinase which performs the PMCA4 tyrosine phosphorylation. These kinases are known to be involved during capacitation (Roa-Espitia *et al.* 2016), implying that PMCA4 could also be regulated by tyrosine phosphorylation in this process. We tested this hypothesis using an immunoprecipitation technique of the tyrosine-phosphorylated proteins during the course of capacitation and showed that PMCA4 was tyrosine-phosphorylated in a basal state, i.e., at t0 of capacitation, and that this phosphorylation did not evolve during capacitation. PMCA4 does not seem to be regulated by tyrosine phosphorylation during mouse sperm capacitation.

Dyslipidemia alters capacitation-associated VDAC2 tyrosine phosphorylation

VDAC2 is another interesting candidate to explain the sperm $[Ca^{2+}]_i$ alterations. It is a voltage-dependent anion channel which forms pores originally identified in mitochondrial outer membranes of eukaryotic cells (Schein *et al.* 1976). VDACs have also been characterized in the plasma membrane of different cell types (reviewed by Shoshan-Barmatz *et al.* (2009)). VDAC2 was localized by immunofluorescence on the plasma membrane of the sperm head and flagellum in human (Liu *et al.* 2011), mouse (Arcelay *et al.* 2008) and bovine (Hinsch *et al.* 2001). One of its functions is to transport calcium either into the cytoplasm or into the mitochondrial intermembrane space (Gincel *et al.* 2001). Its precise role in male fertility is not elucidated yet, but different studies have shown its importance in $[Ca^{2+}]_i$ regulation (Paradowska *et al.* 2006, Liu *et al.* 2011). VDAC2 was also shown as involved in the calcium influx regulation during mouse sperm capacitation (Kwon *et al.* 2013) as its pharmacological inhibition limited the efficiency of this process. Moreover, VDAC2 is known to be tyrosine-phosphorylated during capacitation in human

(Ficarro *et al.* 2003), buffalo (Jagan Mohanarao & Atreja 2012) and mouse (Arcelay *et al.* 2008). In agreement with the literature, we showed that VDAC2 tyrosine phosphorylation had a tendency to increase during capacitation in wt sperm. This mechanism was altered in *Lxra*; $\beta^{-/-}$ -HCD sperm, with a higher basal level of VDAC2 tyrosine phosphorylation compared to wt sperm at t0 (Fig. 6). The link between VDAC2 tyrosine phosphorylation and its activity as a calcium channel is still not clear. However, as shown in several species, one could assume that the increase in VDAC2 tyrosine phosphorylation during the capacitation process could favor the opening of this channel and the rise in $[Ca^{2+}]_i$. We thus propose that the higher $[Ca^{2+}]_i$ in *Lxra*; $\beta^{-/-}$ -HCD sperm at t0 could be due to a higher VDAC2 activity (in relation to a higher tyrosine phosphorylation level) associated with a low quantity of PMCA4. During the course of capacitation, other channels, such as Catsper, are normally activated, and the capacitated cells reach a normally high $[Ca^{2+}]_i$. In *Lxra*; $\beta^{-/-}$ -HCD animals, fewer spermatozoa have the ability to undergo the normal capacitation process, as their epididymal maturation leads to an abnormal membrane composition and dynamics. This study also showed, for the first time, the presence of VDAC2 in mouse epididymosomes, which implies that it may also be transferred to spermatozoa during the epididymal transit, emphasizing its role in the capacitation process.

In conclusion, in our model, dyslipidemia-induced infertility could reside at least partially in the alteration of the acquisition of Ca^{2+} -regulating proteins during sperm maturation via epididymosomes, consequently altering sperm capacitation. Lipids seem to play a central role in this process either by interfering with sperm membrane maturation, epididymosome production and composition, epididymosome-sperm interaction or all these parameters together. Further investigations will be needed to test these hypotheses. Epididymosomes appear as central elements for post-testicular maturation and are highly sensitive to metabolic disorders. Finally, characterization of sperm-related molecules associated with dyslipidemia-induced infertility, such as PMCA4 or VDAC2, may introduce new areas of research to better understand unexplained male infertility associated with undiagnosed dyslipidemia.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0467>.

Declaration of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of the present research.

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