OXYSTEROL NUCLEAR RECEPTOR LXR\(\beta\) REGULATES CHOLESTEROL HOMEOSTASIS AND CONTRACTILE FUNCTION IN MOUSE UTERUS

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ABSTRACT

Uterus is an organ where lipid distribution plays a critical role for its function. Here we show that nuclear receptor for oxysterols LXR\(\beta\) prevents accumulation of cholesteryl esters in mouse myometrium by controlling expression of genes involved in cholesterol efflux and storage (ABCA1 and ABCG1). Upon treatment with an LXR agonist that mimics activation by oxysterols, expression of these target genes was increased in wild-type mice while, in basal conditions, \(lxr\alpha\beta-/-\) mice exhibited a marked decrease in \(abcg1\) accumulation. This change resulted in a phenotype of cholesteryl ester accumulation. Besides, a defect of contractile activity induced by oxytocin or PGF2\(\alpha\) was observed in mice lacking LXR\(\beta\). These results imply that LXR\(\beta\) provides a safety valve to limit cholesteryl ester levels as a basal protective mechanism in the uterus against cholesterol accumulation and is necessary for a correct induction of contractions.

Uterus is schematically divided into two distinct zones: endometrium and myometrium. The endometrium, located in the inner part of the organ, is the site of blastocyst implantation and its epithelium undergoes cyclic radical changes under the control of ovarian sex steroid hormones (1): estrogens are responsible for epithelial cell hyperplasia,
while progesterone blocks cell proliferation and induces differentiation. The myometrium (2), in the outer part of the uterus, accounts for more than 60% of the whole organ (3) and has a primordial role in uterine function. Whereas muscle quiescence due to high progesterone levels is essential during most of the pregnancy (4), efficient myometrium contractility is fundamental for a normal labor (for a review see (5)). This switch results from a modification in the plasma ratio estrogens/progesterone signal that acts as primary event of the parturition. These hormonal changes also induce an increase in the level of endometrial prostaglandins, which play a role in the initiation and maintenance of labor, acting via specific relaxatory or contractile receptors on myometrium initiating contractions (6). Interestingly, it has also been demonstrated that increased production of surfactant protein A by the fetal lung near term causes activation and migration of fetal amniotic fluid macrophages to the maternal uterus, where increased production of IL-1beta activates NF-kappaB, leading to labor (7,8). The inversion of the estradiol/progesterone ratio induces the expression of the oxytocin receptor (OXTR)\(^1\). When activated by oxytocin, a neuropeptide produced by the pituitary, this receptor has a primordial contractile activity on the myometrium during labor. Besides lipid distribution in myometrium is modified during pregnancy in human. While no change in total phospholipids occurs during pregnancy (9), modifications in membrane fluidity take place. Hence transfers of omega 3 and omega 6 polyunsaturated fatty acids, essential for normal fetal growth and development, from the mother to the fetus have been suggested (10). Likewise an increase in local and circulating cholesterol concentrations is observed (11). Even though the role of this plasma cholesterol increase is not clear, apart the anabolic support for the fetus (12), it has been clearly established that this molecule is essential to modulate membrane receptor activity and stability, especially those of OXTR. Indeed Gimpl and Fahrenholz (13) observed an enrichment of oxytocin receptors in cholesterol-rich plasma membranes in HEK 293 fibroblast stably expressing the human oxytocin receptor. In addition, cholesterol stabilizes the receptor in a high affinity state for agonists and protects it from thermal denaturation (for a review see (14)). Smith et al. (15) showed that an abnormal increase in the cholesterol content of uterine smooth muscle cells reduces the amplitude of contractions induced by oxytocin in rat. Moreover, cholesterol depletion with methyl-beta-cyclodextrin could increase the contractions of myometrium strips isolated from rat (15) or guinea pig (16).

Cholesterol and its derivatives are vital nutrients that may also have a major impact on gene expression and thus their intracellular quantities must be tightly regulated. Among the various transcription factors involved in these regulations, Liver X Receptor alpha (LXR\(_\alpha\), NR1H3) and beta (LXR\(_\beta\), NR1H2) play a central role (for a review see (17)). They belong to a subclass of nuclear receptors that form obligate heterodimers with 9-cis retinoic acid receptors (RXR)\(^1\) and are bound to and
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activated by a class of naturally occurring oxysterols (18,19). In absence of ligand, the RXR/LXR heterodimer is constitutively linked to specific DNA target sequences and interacts with corepressors, thus blocking transcription initiation (20,21). The use of LXR-deficient mice (lkr-/-) has also helped to elucidate the role of these nuclear receptors in various physiologic functions (Beaven and Tontonoz, 2006) and many target genes have been described such as the ATP-binding cassette transporters (ABC) A1 (22-24), ABCG5 and ABCG8 (25), responsible for the cholesterol cellular efflux, and the sterol response element binding protein 1c (SREBP1c) involved in lipid metabolism (26).

In this paper we demonstrate that LXRβ functions in the uterus as a sensor to prevent accumulation of cholesteryl esters by coordinately regulating expression of genes encoding proteins involved in cholesterol efflux (ABCA1, ABCG1). Hence, mice lacking LXRβ present an abnormal and specific accumulation of cholesteryl esters in uterine myocytes. Besides, these animals show defects in induced-contractile activity in uterus.

Experimental procedures

Animals - Lxr-knockout mouse (lkrα/-, lkrβ/- and lkrα;β/-) and their wild-type controls were maintained on a mixed strain background (C57BL/6:129Sv) and housed in a temperature-controlled room with a 12 hrs light/dark cycle (27). All experiments were performed on age-matched female mice. For all studies shown, mice were fed ad libitum with water and Global-diet® 2016S from Harlan (Gannat, France) containing 16% protein, 4% fat, 60% carbohydrates. For all experiments, except for contractile activity assays and the mice used for experiments shown in figure 6, animals were treated with a superovulation protocol: intraperitoneally injection of 7 IU pregnant mare's serum gonadotropin on day 1, 5 IU human chorionic gonadotropin on day 3 and sacrificed on day 5 at the end of meta-estrus. For real-time PCR (qPCR) experiments, mice were gavaged with 45 mg/kg T0901317 (T1317) (Cayman Chemical, Montigny le Bretonneux, France) or vehicle (methyl-cellulose) as previously described (28). For contractile activity assays and the mice used for experiments shown in figure 6, estrus was induced with a single injection of 10 µg estradiol benzoate (Sigma-Aldrich, L’Isle D’Abeau, France) 18 hrs before sacrifice. To reduce the effect of stress, the elapsed time between the capture of a mouse and its sacrifice was under 30s. In some experiments, uteri were longitudinally cut and the mucosa gently scraped, as previously described for the intestine (29). Both mucosa and muscular parts were stored in liquid N2 for RNA extraction. All aspects of animal care were approved by the Regional Ethics Committee (authorization CE1-04).

Anatomy and pathology analyses - Uteri from 3 month-old mice were collected, fixed and embedded in paraffin, and 5 µm-thick sections were prepared and stained with Haematoxylin/Eosin/Safran. Lipid staining of
each organ collected was performed on 8 µm-thick cryosections with 1,2 propanediol (Sigma-Aldrich) for 1 min and in oil red O (Sigma-Aldrich) for 4 min as described (30). Cross sectional area of the various parts of the uteri (circular and longitudinal muscular layers, and endometrium) were quantified using Axiovision 4.2 sofware (Carl Zeiss Vision GmbH, Le Pecq, France).

For semi-thin sections, chemicals were from Sigma-Aldrich and Agar Scientific (Saclay, France). Uteri were fixed in 1.2% (v/v) glutaraldehyde buffered in 0.07M sodium cacodylate at pH 7.4 containing 0.05% (w/v) ruthenium red for 1h at room temperature. Samples were post-fixed with 1% (v/v) osmium tetroxide in the same buffer devoid of ruthenium red for 1 hr. Organs were then dehydrated in ethanol baths and propylene oxide (3 times for 20 min), and embedded in propylene oxide and epon epikote resin (v/v) overnight and in epon twice for 3 hrs. Resin polymerisation was conducted at 60°C for 72 hrs. Semi-thin sections (0.8 µm) were cut with a diamond knife (Leica Ultracut S, Rueil-Malmaison, France), and stained with azure 2 followed by addition of NaOH 1N to stop the reaction.

**Analysis of lipid content** - Lipids were extracted as described (31) and analyzed on HPTLC plates. Free cholesterol and cholesteryl esters were identified and quantified against standards by densitometry (Sigma Scan Pro, Sigma-Aldrich) as previously described (27).

**Real-time PCR** - Total RNA was isolated using the Trizol method (Invitrogen, Cergy Pontoise, France) according to the manufacturer’s instructions. cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Charbonnières, France) and random hexamer primers (Promega) according to manufacturer’s recommendations. The real-time PCR was performed on an iCycler (Biorad, Marnes-la-Coquette, France). Four µl of 1/50 diluted cDNA template were amplified by 0.75 U of HotMaster Taq DNA Polymerase (Eppendorf, Brumath, France) using SYBR Green dye to measure duplex DNA formation. Primers are given on Table I.

**Western blot analysis** - Protein extracts (30 µg) from whole uterus were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham-Pharmacia Biotech). Membranes were incubated overnight at 4°C with primary polyclonal antibodies raised against either ABCA1 (1:500; Novus Biological, Montluçon, France), ERα (1:10000; Santa Cruz Biotechnology, Santa Cruz, CA) ERRα (1:200; Santa Cruz Biotechnology), PR (1:200; Santa Cruz Biotechnology), non-cleaved (nc) or cleaved (c)-SREBP1c (1:500 and 1:400; Santa Cruz Biotechnology), SCD1 (1:200; Santa Cruz Biotechnology), PGF2R (1:500; Cayman Chemical) or β-actin (1:2000; Santa Cruz Biotechnology) followed by a 1 h incubation with a peroxidase-conjugated anti-rabbit or anti-goat IgG (1:10000 or 1:5000 respectively; Sigma). Peroxidase activity was detected using the Western Light System (PerkinElmer Life Sciences, Courtaboeuf, France). Protein fold-changes were measured by densitometry of the X-ray films using Quantity One 4.6.1 software (Biorad).
Measurement of uterus contractions in vitro - Uteri were quickly dissected and carefully cleaned of surrounding fat prior to be suspended in organ baths (50 ml) filled with a Dejalon solution (155 mM NaCl, 5.7 mM KCl, 0.55 mM CaCl₂, 6.0 mM NaHCO₃, 2.8 mM glucose, pH 7.4) equilibrated with air and kept at 37°C as described (32) for measurement of tension. A resting tension (2 g) was applied to the suspended uteri. Contractions were recorded with a force-displacement transducer (MyographESAO® 4, Jeulin, Evreux, France) and analyzed with Sérénis® software (Jeulin). Uteri were incubated with increasing concentrations of synthetic oxytocin (Syntocinon®, Novartis Pharma, Rueil-Malmaison, France) or luprostiol, analogous of PGF₂α (Prosolvin®, Intervet, Angers, France). Results are expressed as a dose-response curve showing the uterine tension subtracted of the basal tension.

Statistical Analysis - For statistical analysis, Student’s t test was performed to determine whether there were significant differences between the groups. A p value of 0.05 was considered significant.

RESULTS

Loss of LXRβ results in perturbations of lipid content in uterus

No significant difference in the somatic indexes of uteri was observed among the genotypes of the wild-type (0.38 % +/- 0.03, n=5), the lxra-/- mice (0.37 % +/- 0.04, n=4), the lxrβ-/- mice (0.40 % +/- 0.02, n=7) and the lxra;β-/- mice (0.41 % +/- 0.01, n=5) at 3 months of age. Gross examination of uterus sections from lxr-deficient mice did not reveal any perturbation of the structures as assessed by the presence of an apparently normal endometrium, characterized by a monolayer of epithelial cells and a stroma, and the presence of circular and longitudinal layers of smooth muscle in myometrium (Fig 1A a to d). Uterus structure remains stable even after 12 months of age in all genotypes (data not shown). Determination of the cross sectional area of the smooth muscle pointed out no significant variation in the various knock-out mice compared to the wild-type (Table II). Higher magnification (x 400) did not reveal any perturbation of the endometrium structure (data not shown) whereas vacuoles were visible in layers of myometrium from lxrβ-/- (Fig 1A-g) and lxra;β-/- mice (Fig 1A-h), localized in the cytoplasm of myocytes (Fig 1A). Because LXRs are known to have an important role in the regulation of lipid metabolism in various tissues, we examined whether some differences between wild-type and LXR-deficient mice in uterus lipid content were present. Histological analysis using oil red O staining performed on frozen sections pointed to an abnormal accumulation of neutral lipids in vacuoles observed in myometrium of lxrβ-/- (Fig 1A-k) and lxra;β-/- (Fig 1A-l) mice while no difference among the various genotypes was seen in the endometrium (data not shown). This lipid accumulation was visible in LXRβ-deficient mice as young as 1 month-old (data not shown). Since lxra-/- mice appeared to have no lipid-rich vacuole (Fig
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1A-j), we concluded that the phenotype was due to the absence of LXRβ.

Semi-thin sections (0.8 µm) of osmium tetroxide-fixed uteri were performed to precisely determine the localization of these vacuoles. Azure 2 dye, which stains lipids in yellow, showed that these vacuoles were localized in the cytoplasm of myocytes (Figure 1B) and did not result of an infiltration of adipose tissue within the smooth muscle, as also suggested by the absence of any significant increase levels of adipocyte marker mRNA such as the fatty acid binding protein (aP2) and peroxisomal proliferator activated receptor (PPARα and PPARγ) (figure 4A).

In order to determine whether this lipid accumulation was generalized to various muscles, oil red O staining was performed on frozen slides of three different types of muscle: intestine (duodenum), heart and rough muscle (quadriceps) of 3 month-old wild-type and lxrα;β−/− females (Figure 1C). None of the tested tissues were stained positively, except uterine smooth muscle which was used as a control. These data led us to suggest the existence of tissue-specific mechanisms by which LXRβ regulates lipid homeostasis in uterine smooth muscle.

LXRβ null mice have elevated uterus cholesteryl esters

To determine the nature of lipids accumulated in the uterus, thin layer chromatography analyses were performed on whole lipid extracts from uteri of 3 and 12-month old mice. While LXR-mediated triacylglycerols accumulation had already been reported in vascular smooth muscle cells (33), biochemical analysis revealed that only the fraction containing cholesteryl esters was significantly increased after normalizing to uterus weight at 3 months (23.5- and 37.2-fold in lxrβ−/− and lxrα;β−/− mice, compared to wild-type mice; p<0.0005) and 12 months of age (27.6- and 66.5-fold in lxrβ−/− and lxrα;β−/− mice, compared to wild-type mice; p<0.0005) (Figure 2). Lxra−/− mice presented the same low amount of cholesteryl esters as the wild-type mice. Together, these data suggest that the increase in the oil red O staining observed in the lxrβ−/− and lxrα;β−/− uterus was due to the accumulation of cholesteryl esters. Whatever the age considered, cholesteryl ester concentration was significantly higher in lxrα;β−/− uterus than in lxrβ−/−. This could suggest a mechanism of a slight redundancy between the two isoforms, where LXRα could partially reverse the drastic phenotype induced by absence of LXRβ. No significant differences in free cholesterol (Figure 2, white bars), triacylglycerol and phospholipid contents (data not shown) were observed among the genotypes.

Both LXRα and LXRβ are expressed in the various compartments of uterus

The results described above suggested that the LXR-dependent changes observed in the cholesteryl esters content were primarily due to LXRβ. We wondered whether this specificity in the LXR isoform was due to the exclusive expression of LXRβ in the tissue. Presence of LXRα and LXRβ mRNA were checked by qPCR1 on whole uterus as well as mucosa and
muscular parts. As shown in figure 3, *lxra* and *lxrb* were detected in myometrium and endometrium parts of the uterus. In the myometrium, both isoform mRNAs were in a comparable amount compared to whole uterus. In order to confirm that we had an enrichment of the muscular part, OXTR mRNA was amplified and the highest accumulation was expectedly obtained in the myometrium fraction.

**LXRs regulate cholesterol efflux and fatty-acid metabolism in uterus in vivo**

To explore the underlying molecular mechanisms that might account for the cholesteryl ester accumulation in LXRβ-deficient mice, gene expression was examined by qPCR from whole uteri of wild-type and *lxra;β−/−* animals gavaged with the potent synthetic LXR agonist T1317 (Figure 4A). In both genotypes, basal and T1317-induced levels of genes encoding scavenger receptor BI receptor (SRBI) involved in cell cholesterol entry, 3-hydroxy-3-methylglutaryl-Coenzyme A (HMGCoA) reductase (*red*) and synthase (*syn*), responsible of de novo cholesterol synthesis, and acyl-Coenzyme A: cholesterol acyltransferase (ACAT)1 and 2, implicated in cholesterol esterification, were unchanged in both genotypes. In contrast, expression of *abca1* and *abcg1*, encoding two cholesterol efflux transporters, showed a LXR-dependant regulation. T1317-treatment induced an increase of *abca1* and *abcg1* accumulation in uteri from wild-type mice (2.7- and 5.2-fold increase, respectively; *p*<0.01). No induction of the LXR-target genes was seen in *lxrb*-deficient mice. As expected, a higher accumulation of ABCA1 was observed in the T1317-treated wild-type mice (5-fold compared to the vehicle-treated animals, *p*<0.01) (Figure 6B).

Not surprisingly, transcripts of the low density lipoprotein receptor *ldlr* was significantly lower in *lxra;β−/−* mice since this gene is known to be regulated by the intracellular concentration of oxysterols through a negative regulation loop (34). Interestingly, while the basal level of *abca1* was unchanged, basal accumulation of *abcg1* was significantly lower in *lxra;β−/−* females (66% less that the wild-type mice, *p*<0.05; Figure 4A). It is assumed that this decrease could be considered as the primum movens of the phenotype, since the intracellular cholesterol increase cannot induce ABCA1 and ABCG1 transporters leading to its sequestration and accumulation in myocytes. It could thus be suspected that LXRs regulate cholesterol efflux in uterus myocytes.

Besides, RNA accumulation of known target genes of LXRs involved in fatty acid metabolism was studied (Figure 4B). T1317-treatment induced the accumulation of *srebp1c* (31.3-fold, *p*<0.005) and *lpl* (2.6-fold, *p*<0.01) in wild-type mice, encoding respectively SREBP1c and the lipoprotein lipase (LPL). Quite surprisingly, level of non-cleaved (nc-SREBP1c) form of SREBP1c did not appear to be different among the genotypes whatever the treatment (Fig 6B). Likewise no variation of the cleaved form (c-SREBP1c) was observed in the same samples (Fig 6B). Interestingly, the gene encoding the fatty acid
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synthase fasβ, which has been shown to be a LXR-target gene, is basally less expressed in lxra;β−/− females compared to the wild-type, and not induced by T1317 in the wild-type mice. Scd1 and scd2, encoding stearoyl coA-desaturase 1 and 2, show a higher accumulation in the uteri from T1317-treated wild-type mice (4.0- and 1.7-fold respectively, p<0.05). Even not significant, a higher protein level of SCD1 (3-fold) was observed only in the wild-type mice after the T1317-treatment (Fig 6B), while SCD2 was undetectable in all the samples (data not shown). These results suggested that, as already described in other tissues, LXRs might be involved in the regulation of fatty acid homeostasis in uterus.

**Uteri of LXRβ-deficient mice present contraction defects**

Since myometrium is the major actor during labor, we investigated whether the cholesteryl esters accumulation could modify uterine contractile activity. Assays were thus performed on uteri from the various genotypes in order to measure the response of the muscle to either oxytocin or luprostiol, a PGF2α analog. Uterus from wild-type and lxra;β−/− females presented a similar increase of contraction amplitudes when oxytocin (Fig 5A) or luprostiol (Fig 5B) were added in increasing concentrations in the media. While uterus from lxrβ−/− and lxra;β−/− mice presented no difference compared to the wild-type mice in the basal contractions (Fig 5), the organs were less responsive to higher concentrations of oxytocin and PGF2α analog (p<0.01 compared to the wild-type and lxra;−/− mice, n= 6 to 9).

Interestingly the measured efficient doses of oxytocin and luprostiol to induce the maximal amplitude of contraction were identical in all genotypes (5x10^−3 U/ml and 3x10^−4 mg/ml for oxytocin and luprostiol, respectively). Even though these data suggested that the amount of receptors was not affected, we analyzed by qPCR the levels of OXTR and PGF2α receptor (PGF2R). As shown in figure 6A, no basal variation was observed between the wild-type and lxra;β−/− mice; besides, T1317 gavage had no significant effect on OXTR and PGF2R RNA levels, as well as on PGF2R protein accumulation (Fig 6B). Likewise, because mice were synchronized by the intraperitoneal injection of E2, we hypothesized that the estradiol dependent activities, which regulate the expression of various genes such as OXTR, could have been altered in the engineered mice. QPCR analyses showed that contraction impairment was not due to basal neither induced decrease in mRNA or protein levels of estrogen (ERα) and progesterone (PR) receptors, and estrogen-related receptor α (ERRα) (Fig 6A&B). Altogether, these data suggested that the phenotype observed in mice lacking LXRβ was probably more due to a muscular defect than to a drastic steroid hormone signaling defect, as pointed by the significant decrease of SM22alpha encoding transgelin, a calponin which is expressed exclusively in smooth muscle-containing tissues of adult animals (65% of decrease in the lxra;β−/− mice compared to the wild-type
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mice; p<0.05). SM22alpha is one of the earliest markers of differentiated smooth muscle cells.

DISCUSSION

In this report we detail the discovery of LXRβ as an important regulator of cholesterol homeostasis in uterus through its ability to modulate transcription of genes encoding proteins that regulate cholesterol efflux (ABCA1, ABCG1) and fatty acid synthesis (LPL, SREBP-1c, SCD1/2). Besides, the uteri of mice lacking LXRβ present an abnormal capacity to contract under oxytocin or PGF2α signals. LXRβ appears thus to provide a cholesterol safety valve that operates as in other tissues as a sterol sensor and thereby maintains the concentration of free cholesterol below toxic levels. In mice lacking LXRβ, LXRα does not present a totally redundant function.

LXRβ regulates cholesterol efflux within the myocytes

The ability of LXRs to control muscular lipid metabolism is reaching a high interest state. Studies (35,36) showed that LXR activation can promote triglyceride accumulation in the presence of high glucose concentration in skeletal muscle cells, via the induction of the expression of lipogenic enzymes such as SREBP1c (26), FAS (37) and SCD1 (38). In parallel, LXRs have been described as regulators of cholesterol efflux from the rough muscle by increasing the efflux of intracellular cholesterol to extracellular acceptors such as high density lipoprotein (39). Despite these data, little was known about the role of these nuclear receptors in smooth muscle. Davies et al. (33) pointed out that T1317 could induce triacylglycerol accumulation in human vascular smooth muscle cells by activating FAS, SREBP-1c and SCD-1. We found that mice lacking LXRβ presented a high accumulation of cholesteryl esters in longitudinal layers as well as circular layers of myometrium. Since abcg1 is a target gene of LXRs in uterus, as shown by the induction of the mRNA accumulation after the gavage of wild-type mice with T1317, and because its basal level was significantly lower in mice lacking LXRβ, we hypothesized that LXRβ is a central sensor of cholesterol status of the uterine myocytes. Moreover this role seems to be specific of the uterus since the other muscles from lxrβ-/- mice tested so far (heart, duodenum, quadriceps) did not show such profile of oil red O staining. Analysis of the molecular mechanisms leading to such specificity would be of interest in order to screen whether expression of cofactors within these organs could in part explain the phenotype.

Moreover, as in other tissues, lipogenic genes are also regulated by LXRs. Whether srebp1c, lpl and scd1/2 are specific LXRβ-target genes in uterus has not been determined yet. The status of SCD1 and SCD2 is of particular interest. Indeed these enzymes are responsible for the ∆9-cis desaturation of stearoyl- and palmitoyl-CoA, producing oleoyl- and palmitoleoyl-CoA. Oleoyl-CoA is
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the substrate for cholesterol acyltransferase and enables more esterification of cholesterol. However, no significant basal change of these enzymes was observed in the \(lxra;\beta^{-}\) mice. Besides, no clear defect in triglyceride concentration was detected in uterus even though \(fas\) presented a lower basal level in \(lxra;\beta^{-}\) mice.

**LXRβ deficiency leads to uterus contraction defect**

The decreased total amplitude of contractions did not seem to be due to a loss of muscle mass as pointed by the cross sectional area analysis in the LXRβ-deficient mice and the somatic index, but rather to a muscular defect as suggested by the decrease of SM22alpha transcript. Besides, even though a direct role of LXRβ in the control of the contractile activity of uterus could not be ruled out, several lines of evidence suggest that cholesterol could modulate contractile activity of various smooth muscles. Pharmacological depletion of cholesterol by methyl-β-cyclodextrin abolished induced contractions of ureter and portal vein (40), and arteries (41) in rat. Conversely, Smith et al. (15) showed that cholesterol inhibited uterus contraction of pregnant rat by destabilizing caveolae. Altogether these data prove that cholesterol concentration has dramatic effects on smooth muscle contraction and that in uterus, cholesterol amount is negatively correlated to its ability to contract. Consistently, our results show that LXRβ-deficient mice, which presented an increased cholesteryl ester concentration, exhibited a lower capacity to contract under stimulation with oxytocin and PGF2α analog.

It is interesting to note that even though \(lxrβ^{-}\) and \(lxra;\beta^{-}\) mice apparently deliver successfully pups at term, LXRβ-deficient females usually show various signs of fetal resorption in the uterine horns. In some cases, 3 to 9 month-old \(lxrβ^{-}\) and \(lxra;\beta^{-}\) females develop hind-leg paralysis few days or weeks after delivery. When necropsy of these females is done, non-expulsed pups could be observed in uterine horns (Figure 7A). In rare cases, the female died giving birth (Figure 7B). Hence, it could be hypothesized that LXR-signaling pathway is one of the actors responsible of lipid change in plasma membrane of uterus myocytes at the term of pregnancy, thus initiating the ability of uterus to properly deliver the pups. Besides, it has been pointed that obese and overweight women often have dysfunctional labor. Obesity is related to many complications associated with pregnancy, for example: gestational diabetes mellitus, tromboembolic problems and hypertensive disorders such as preeclampsia or eclampsia (42). It has been shown that even with an uncomplicated pregnancy, there was a need for more oxytocin infusion to induce labor in overweight and obese women than in normal weight (42). Moreover, pre-pregnancy body mass index (43,44) and increase in its category (45) during pregnancy have been related to be high-risk factors for cesarean delivery at term of pregnancy for failure to progress in the labor. However, the molecular mechanisms by which obesity and overweight lead to difficult labor
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remain unknown so far. Nevertheless, the lxb-/- females could be considered as the first engineered mice presenting an abnormal contraction of uterus that could be used to understand how disequilibrium in the lipid diet could interfere with a normal parturition in human. Screening of new specific targets of LXRβ would thus be helpful to study side effects of the overweight on labor.

LXRα does not have redundant functions in the uterus

It has been generally admitted that LXRβ is ubiquitously expressed, whereas LXRα expression is limited to tissues where lipid metabolism is high. Actually it looks like that very few tissues do not express LXRβ (46). So far, few physiologic functions have been associated to LXRβ in vivo, since, conversely to lxa-/- mice, lxb-/- mice do not present clear-cut phenotypes. Up to now, only two functions have been reported for LXRβ in mouse. Komuvets et al. (47) were the first to report an alteration in the LXRβ-deficient mice, which presented an abnormal differentiation of the epidermis while LXRα-deficient mice appeared normal. The authors showed that only LXRβ was present in the affected tissue, suggesting that this phenotype could develop because of the lack of LXRα expression and thus the absence of any possible redundancy. In the testis we (Volle et al., submitted data) and others (48,49) described that LXRβ was important for the regulation of the cholesterol metabolism in Sertoli cells. Conversely to epidermis, LXRα is expressed in the Sertoli cells (Volle et al. submitted data). The present data point out a combined phenotype of cholesteryl ester accumulation and lower contraction capacity due to the lack of LXRβ while LXRα is expressed. This fact has to be paralleled to the phenotype observed in the lxa-/- mice fed high-amount of cholesterol where the presence of LXRβ could not rescue the regulation of cyp7a1 (50). Hence, even though both receptors could bind the same DNA sequence in vitro (17), LXRα and LXRβ can differentially regulate gene expression and thus in uterus they clearly do not have overlapping roles. These data strongly support the existence of specific molecular mechanisms leading to LXRβ transactivation such as specific promoter sequences or specific cofactors for each isoform. Such elements remain to be discovered.

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**FOOTNOTES**

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1The abbreviations used: ABC, ATP-binding cassette transporters; ACAT 1 and 2, acyl-Coenzyme A: cholesterol acyltransferases 1 and 2; ERα, estrogen receptor alpha; ERRα, estrogen-related receptor alpha; FAS, fatty acid synthase; HMGCoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; LDL-R, low density lipoprotein receptor; LPL, lipoprotein lipase; LXRα, liver X receptor alpha, LXRβ, liver X receptor beta; lxr−/−, LXR-knockout mice; OXTR, oxytocin receptor; PGF2R, PGF2α receptor; PR, progesterone receptor; qPCR, real-time quantitative RT-PCR; red, 3-hydroxy-3-methylglutaryl-Coenzyme A (HMGCoA) reductase encoding gene; SM22alpha, transgelin encoding gene; syn, 3-hydroxy-3-methylglutaryl-Coenzyme A (HMGCoA) synthase encoding gene; RXR, retinoid X receptor; scd1 and scd2, stearoyl coA-desaturase 1 and 2 genes; SR-B1, scavenger receptor-B1; SREBP-1c, sterol regulatory binding protein-1c; T1317, LXR agonist T0901317.

FIGURE LEGENDS

Figure 1. LXRβ-deficient mice present an abnormal accumulation of neutral lipids in uterine myocytes only.

(A) Histological examination of uteri from wild-type, LXRα- and/or β-deficient mice. a-h: Haematoxylin/Eosine/Safran staining at two magnifications. Squared portions indicate the magnified view; i-l: oil red O staining; bars indicate the different sizes (200 or 20 µm). Black arrowheads indicate the epithelium. Black arrows indicate cytoplasmic vacuoles in myocytes. LL, longitudinal muscular layer; CL, circular muscular layer; E, endometrium. (B) Azure blue 2B staining of semi-thin sections from the muscular layer. Lipids are stained in yellow. Dash line: plasma membrane of a myocyte. Bar: 5 µm. (C) Oil red O staining was performed on frozen sections from wild-type and lxrα;β−/− mice of 3 months of age as described in the material and methods section. Bar: 20 µm.

Figure 2. Uterus from LXR-deficient mice accumulate cholesteryl esters at 3 and 12 months of age.

Analysis of free cholesterol (open bars) and cholesteryl ester (black bars) concentrations were determined as described in the material and methods section from whole uteri. Histograms are indicated as means +/- SEM. N=5, except for lxrα;β−/− at 12 months (N=8). *, p<0.05; ***, p<0.005.

Figure 3. LXRα and LXRβ are expressed in the myometrium and endometrium.

RNAs were prepared from whole uteri and from epithelial and muscular parts of the uterus as described in the material and methods section to determine the levels of mRNA expression of LXRα,
LXRβ and OXTR. Quantification (mean +/- SEM) was done by qPCR (N=4 to 6). Results obtained in the whole uteri was indicated as 1.

**Figure 4: LXRs regulate genes involved in lipid homeostasis in uterus.**
A) Genes involved in cholesterol entry and efflux, and adipocyte markers. B) Genes involved in fatty acid synthesis. Transcripts were quantified by qPCR analysis (mean +/- SEM). N=5 to 8. T1317: T0901317; ldlr, low-density lipoprotein receptor; srbi, scavenger receptor BI; red, HMG-CoA reductase; syn, HMG-CoA synthase; acat1 and acat2, acyl-Coenzyme A: cholesterol acyltransferase 1 and 2; abca1, ATP-binding cassette A1; abcg1, ATP-binding cassette G1; ap2, fatty acid binding protein; ppar, peroxisomal proliferator activated receptor; fas, fatty acid synthase; lpl, lipoprotein lipase; scd1 and scd2, stearoyl coA-desaturase 1 and 2; srebplc, sterol response element binding protein 1c. *: p<0.05 vs. vehicle-gavaged wild-type mice; **: p<0.01 vs. vehicle-gavaged wild-type mice; ***: p<0.005 vs. vehicle-gavaged wild-type mice.

**Figure 5: Uteri from mice lacking LXRβ present a defect of induced contractions.**
Contractile activity was assessed on uteri from estrogen-treated wild-type and lxr-deficient mice. Main amplitude contraction was recorded in response to synthetic oxytocin or PGF2α analog luprostiol. **: p<0.01 vs. wild-type mice; #: p<0.05 vs. lxrα/- mice; ##: p<0.01 vs. lxrα/- mice. Each point represents the mean +/- SEM. N= 6 to 9 animals.

**Figure 6: The decrease in the amplitude of contraction is not linked to oxytocin or PGF2α receptor expression or estrogen signalling pathway defects but to a muscular defect.**
A) RNAs were extracted from uteri of estrogen-treated wild-type and lxr-deficient mice. Transcripts of genes encoding hormone receptors were quantified by real-time PCR analysis (mean +/- SEM). N=5 to 8. B). Representative western blots of proteins extracted from uteri of estrogen-treated wild-type and lxr-deficient mice. N=5. ABCA1, ATP-binding cassette transporter A1; OXTR, oxytocin receptor; PGF2R, PGF2α receptor; SM22alpha, transgelin; ERα, estrogen receptor alpha; ERRα, estrogen-related receptor alpha; PR, progesterone receptor, SCD1, stearoyl coA-desaturase 1; nc- or c-SREBP-1c, non cleaved or cleaved sterol regulatory binding protein-1c.

**Figure 7: In rare cases, non-delivered pups are found blocked in the uterus horn or in the vagina of LXRβ-deficient mice.**
A) Left: necropsy of a 6 month old lxrβ/- mouse presenting an abdominal distension and suffering of hind-leg paralysis two weeks after delivery. Right: necropsy of a 6 month old lxrα;β/- mouse. The right uterine horn was opened. Dashed line, non-expulsed fetus; asterisk, placenta; arrowheads, ovary; H, uterine horn; C, cervix; bar, 0.5 cm. B) Lxrα;β/- female dead during parturition. Note the pup blocked in the cervix/vagina.
Table I. Sequence primers used for QPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’-&gt;3’ sequences</th>
<th>Size of the amplicon</th>
<th>Ref</th>
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<td>106</td>
<td>this study</td>
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<tr>
<td>abcg1 (NM_009593)</td>
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<td>ERα (NM_007956)</td>
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<td>SM22α (U36588)</td>
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Table II. Surface analysis of the cross sectional area of the uteri.

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<th>Genotype</th>
<th>Endometrium</th>
<th>CL</th>
<th>LL</th>
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<tr>
<td>Wild-type (15)</td>
<td>43.5 +/- 1.7</td>
<td>14.6 +/- 0.5</td>
<td>41.9 +/- 2.0</td>
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<td>Lxrα-/- (12)</td>
<td>39.4 +/- 1.2</td>
<td>14.3 +/- 0.8</td>
<td>46.3 +/- 1.7</td>
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<td>Lxrβ-/- (16)</td>
<td>43.0 +/- 3.5</td>
<td>17.1 +/- 1.2</td>
<td>39.9 +/- 2.7</td>
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<tr>
<td>Lxrα;β-/- (15)</td>
<td>43.4 +/- 1.1</td>
<td>14.0 +/- 0.6</td>
<td>42.6 +/- 1.4</td>
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</tbody>
</table>

Quantifications (mean +/- SEM) represent the relative surface of each part of the uterus: endometrium, circular (CL) and longitudinal (LL) layers. Number of analysed cross sections is indicated in brackets.
Mouzat et al. Fig 1A

Mouzat et al. Fig 1B

Mouzat et al. Fig 1C
WT lxrα⁻/⁻ lxrβ⁻/⁻ lxrα;β⁻/⁻

3 months

WT lxrα⁻/⁻ lxrβ⁻/⁻ lxrα;β⁻/⁻

12 months

Free
Esters

Cholesterol (mg/g tissue)

0 2 4 6 8 10 12 14 16 18

3 months

12 months

Mouzat et al. Fig 2

Free
Esters

Relative mRNA accumulation (vs cyclophilin)

0 1 2 3 4 5 6 7

Whole Uterus Myometrium Endometrium

Mouzat et al. Fig 3

Mouzat et al. Fig 2

Mouzat et al. Fig 3
Mouzat et al. Fig 4A

Mouzat et al. Fig 4B
Mouzat et al. Fig. 7
Oxysterol nuclear receptor LXRβ regulates cholesterol homeostasis and contractile function in mouse uterus

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