Modulation of estrogen signaling in hepatic and vascular tissue

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Repression of Hepatic Estrogen Receptor Alpha Does Affect Expression of Lipid-Related Gene but Does Not Affect Lipid Metabolism in Female APOE*3 Leiden Mice.

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Abstract

Estrogens have been shown to modulate the lipoprotein profile. However, the role of the hepatic estrogen receptor α (ERα) in this process is unclear. In the present study, we have addressed the role of hepatic ERα signalling in lipid metabolism of APOE*3 Leiden transgenic mice fed a high fat diet. Hepatic ERα was down regulated using adenovirus-mediated transfer of a short hairpin (sh) RNA directed against the ERα (Ad.shERα). Despite significant down-regulation of hepatic ERα RNA and protein levels (60%), plasma cholesterol, triglyceride and glucose levels were not changed. In addition, no effects on the VLDL-TG secretion rate and intra-hepatic lipid levels were observed. In contrast, expression of the Cyp7a and PPARα genes was up regulated 2- and 2.5-fold, respectively, and the SHP gene was down regulated 2-fold. Apparently, the changes in the expression of these lipid related genes is compensated for by alternative transcriptional or post-transcriptional mechanisms and does not affect plasma lipid levels. In conclusion, repression of hepatic ERα gene expression does affect genes involved in lipid metabolism, but does not have an obvious impact on lipid parameters.

Introduction

Epidemiological studies have shown that the menopausal transition is association with changes in circulating lipid levels, including elevated plasma levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), and reduced levels of high-density lipoprotein cholesterol (HDL-C). Since estrogen treatment has been reported to influence these lipid levels in the opposite manner [1-4], estrogen has been postulated to be beneficial in cholesterol homeostasis.

The estrogenic effects are predominantly mediated via activation of either of two estrogen receptors (ERs), ERα and ERβ. These ligand-dependent transcription factors modulate gene transcription but can also interfere with intracellular signaling pathways [5-7]. To date, mouse models of estrogen deficiency, such as aromatase knockout (ArKO) and ERα and ERβ knockout mice have been used to gain insight into the role of estrogens in lipid metabolism. ArKO mice, ERα - and double ERα/β knockout mice all develop hypercholesterolemia [8-10], whereas no lipid phenotype was described in ERβ knockout mice [10]. These results demonstrate a role for estrogen in lipid homeostasis, and indicate that ERα is involved.

The liver plays a central regulating role in lipid metabolism. To gain insight into the role of hepatic ERα in lipid homeostasis, Ad vectors encoding shRNA's directed against mouse ERα (Ad.shERα) [11] were administered to hyperlipidemic APOE*3-Leiden female mice. Hepatic ERα mRNA and protein levels were repressed by 60%, and were associated with changes in the expression level of genes involved in lipid metabolism. However, plasma lipid parameters were not affected upon Ad.shERα administration. These results indicate that the hepatic ERα level does not play a rate limiting role in lipid metabolism.

Results

Basal body weight and plasma parameters

To induce hyperlipidemia, two groups of female APOE*3-Leiden mice were fed a high fat cholesterol enriched diet (diet W) for eight weeks. After this period, mice in both groups had an average bodyweight of 21 gram, serum glucose levels of 6 mM and triglyceride (TG) level of 1.9 mM. In addition, both groups of mice exhibited hypercholesterolemia (13.4 and 13.8 mM) (table 1).

Table 1. Bodyweight and glucose levels in 4 hrs-fasted ApoE*3-Leiden female mice fed a high fat diet, before and after Ad-mediated gene transfer of shERα

	Day 0		Day 5	
	Ad.Empty	**Ad.shERα**	**Ad.Empty**	**Ad.shERα**
Bodyweight (gr)	20.9 ± 1.4	21.2 ± 1.2	20.1 ± 1.2	20.2 ± 1.3
Glucose (mmol/l)	6.1 ± 1.0	5.8 ± 1.1	6.5 ± 0.7	6.6 ± 0.8
Cholesterol	13.4 ± 4.4	13.8 ± 3.3	8.6 ± 0.9	8.6 ± 1.4
Triglycerides	1.9 ± 0.9	1.9 ± 0.5	3.2 ± 0.5	3.1 ± 0.6

Hepatic ERα levels in ApoE*3-Leiden mice after Ad.shERα treatment

To down-regulate the hepatic ERα, the hyperlipidemic female APOE*3-Leiden mice were injected either with Ad.Empty or with Ad.shERα. Five days after Ad.shERα treatment ($1.5.10^9$ pfu), the hepatic ERα RNA and protein levels were repressed by 60% as compared to Ad.Empty treated mice ($P<0.05$, $P<0.001$, respectively; Figure 1A and 1B).

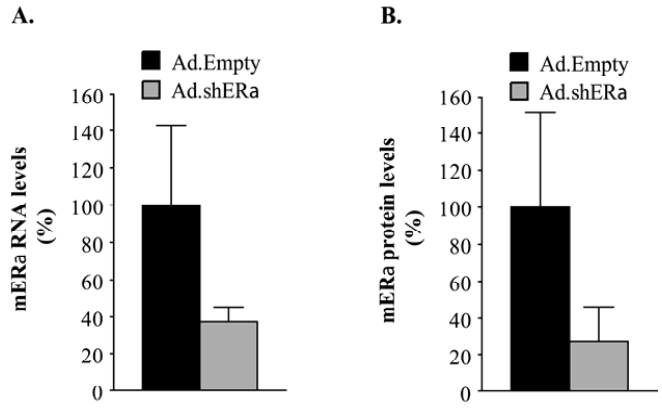

Figure 1. Hepatic ERα levels after Ad mediated transfer of shERα *in vivo*
Female APOE*3-Leiden mice were injected with 1,5.10^9 pfu Ad.Empty or Ad.shERα (n=5). Livers were harvested five days after Ad. administration and subjected to taqman (A) and western (B) analysis. Respectively, HPRT and p38 were used as internal standard. Data represent as mean ± SD

Body weight and plasma parameters in Ad.shERα treated mice

At day five after Ad administration, cholesterol and TG levels were modulated to the same extent in both Ad.Empty and Ad.shERα treated mice (Table 2). Thus, down-regulation of hepatic ERα did not affect serum lipid nor glucose levels.

Hepatic VLDL-TG production

After down regulation of hepatic ERα in APOE*3-Leiden mice, the VLDL-TG production rate was determined by injection of Triton WR1339. Triton WR1339 blocks VLDL-TG lipolysis and VLDL remnant clearance and the increase in plasma TG is a measure for VLDL-TG production. Reduced hepatic ERα levels did not affect the VLDL-TG production rate (Figure 2).

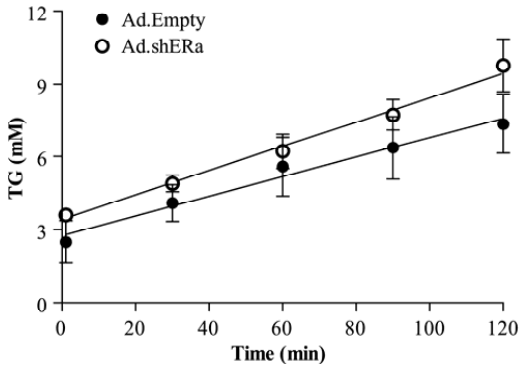

Figure 2. Hepatic VLDL-TG production after Ad mediated transfer of shERα *in vivo*
Female APOE*3-Leiden mice were injected with $1{,}5.10^9$ pfu Ad.Empty or Ad.shERα (n=4). VLDL-TG production was measured 5 days post-injection. Fasted serum TG level was determined between 0 and 120 min after Triton WR 1339 injection. Values are represented as mean ± SD.

Hepatic Lipid content

In addition, hepatic TG and Chol content were analyzed. As depicted in Figure 3, the hepatic lipid content of the Ad.shERα treated APOE*3-Leiden mice did not differ from the Ad.Empty treated group (Chol; 14.4 ± 1.7 versus 13.8 ± 2.9, for TG; 104.5 ± 38.7 versus 93.1 ± 31.9, for Chol esters; 33.8 ± 7.6 versus 29.9 ± 6.2 mM, respectively).

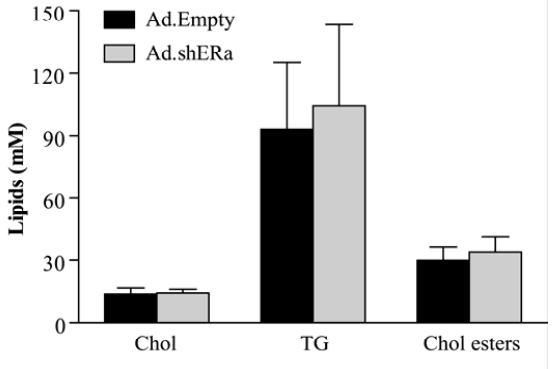

Figure 3. Hepatic lipid content after Ad mediated transfer of shERα *in vivo*
Hepatic TG and cholesterol content was analyzed in APOE*3-Leiden female mice five days after Ad.Empty and Ad.shERα administration. Values represent the mean±SD of 11 mice.

Hepatic mRNA expression levels

To further investigate the effect of short-term repression of ERα in liver, hepatic expression of genes involved in lipogenesis were assessed by real-time PCR. Short-term repression of hepatic ERα led to a significant enhancement of peroxisome proliferator-activated receptor

(PPAR)α and Cyp7α and a significant repression of short heterodimer partner SHP (Fig 4). Transcription levels of apolipoprotein E (ApoE) and ApoAV were unchanged (Fig 4).

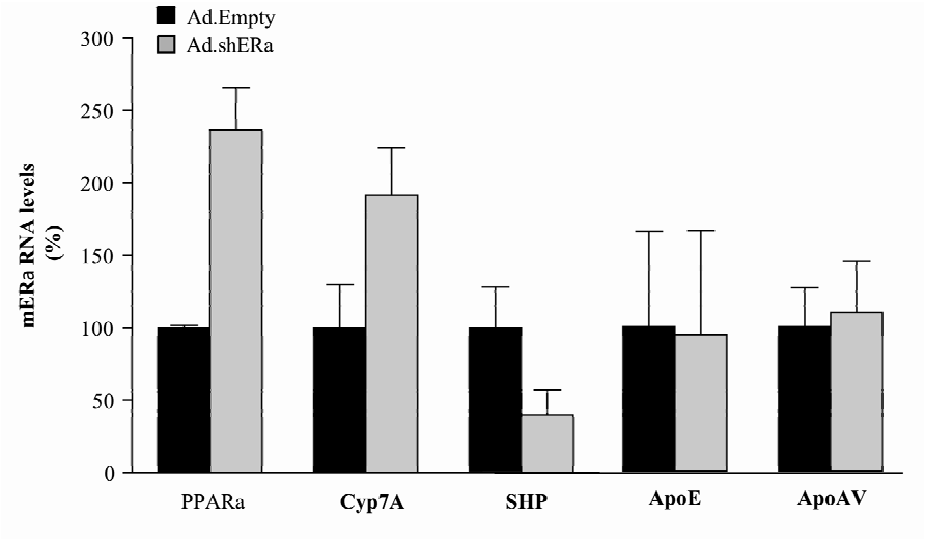

Figure 4. Hepatic gene expression after Ad mediated transfer of shERα *in vivo*
Gene expression was analyzed by real time PCR in APOE*3-Leiden female mice five days after Ad.Empty and Ad.shERα administration. The HPRT gene was used as internal standard. Values represent the mean±SD (n=5) relative to the percentage of expression in Ad.Empty treated mice. *, statistically significant difference of $P<0.05$ compared with Ad.Empty treated mice.

Discussion

The present study evaluates the direct role of hepatic ERα in lipid homeostasis. To this end, hepatic ERα was down-regulated in hyperlipidemic APOE*3-Leiden female mice using Ad mediated transfer of a shRNA construct targeted against the ERα. This resulted in a 60% reduction in hepatic ERα RNA and protein levels and significant changes in PPARα, Cyp7α and SHP gene transcription. However, hepatic lipid levels and serum lipid and glucose levels were not affected by ERα down-regulation. Apparently, the hepatic ERα is involved in regulating hepatic gene transcription, but ERα level does not play a rate limiting role in determining serum lipid or glucose levels in hyperlipidemic APOE*3Leiden mice.

The application of vector-based systems expressing small hairpin RNA (shRNA) to dissect gene function is now well established in mammalian cells in vitro. In vivo application requires highly efficient delivery of the shRNA expression construct and for this, in the current paper Ad vectors are used. These have been shown to efficiently knock-down ERα in

liver in vivo, resulting in highly efficient reduction of ERα transcriptional activity [11]. Ad vectors predominantly transduce hepatic parenchymal cells, which also most abundantly express ERα (data not shown) [12,13]. We were able to repress hepatic ERα RNA and protein levels to an extent of 60% with a moderate viral dose ($1,5.10^9$ pfu/mice). At higher viral dosages, the reduction in gene expression was not further increased and hepatotoxicity did occur (data not shown).

We previously demonstrated that the shERα construct used here is specific for the murine ERα [11]. Since the ERβ is expressed at very low levels in parenchymal cells, and our shRNA construct harbours nine mismatches with the murine ERβ, it seems unlikely that off target effects explain the effect of shERa expression on hepatic gene expression. However, we cannot exclude that some of the effects on gene expression are mediated by the so called a-specific interferon response to double stranded RNA. However, it is likely that the Ad transduction per se induces a much more dramatic cellular stress response as compared to the dsRNA. This ad specific effect was controlled for in the comparison with the ad empty treated groups.

The reduction in ERα level is associated with significant changes in the expression of genes involved in lipid metabolism. The Cyp7α gene encodes the enzyme controlling the first and rate-limiting step in cholesterol degradation to bile acids. Upregulation of the Cyp7α gene is in line with the observed down regulation of SHP, as SHP negatively regulates expression of Cyp7α. Interestingly, SHP appears to be induced by estrogen in liver of wt mice [14,15], and a decrease of estrogen signaling would thus be in line with SHP downregulation. Agonists of the transcription factor PPARα prevent lipid accumulation in liver by stimulating fatty acid β-oxidation in liver [16,17] a process which is also found to be induced by estrogens [18]. The upregulation of PPARa in response to a reduction in Era could therefore be a compensatory effect. Since we did not find a lipid phenotype associated with these gene expression changes, it seems likely that the gene expression changes them selves or post-transcriptional regulatory events counterbalance each other. Apparently, the liver can compensate for changes in estrogen signaling in such a manner as to maintain normal plasma and liver lipid levels.

The role of ERα in lipid metabolism has been addressed using whole-body knockout mouse models lacking ERα. These ERα knockout mouse models as well as the aromatase knock out mouse model, which lacks the final step in estrogen synthesis, display a lipid phenotype that is apparent upon aging [10,19,20]. The phenotype associated with any

knockout mouse model needs to be considered with the provision that compensatory changes that counteract some of the knockout effects may have occurred. The delayed lipid phenotype of the ERα knockout and ARKO mouse models could be explained by a failure of this compensation in time. By reducing the hepatic ERα mediated signalling cascade during adulthood and assessing the parameters relatively soon thereafter, our data indicate that the lack of a lipid phenotype of ERα knock outs is not due to compensatory changes. In stead, we conclude that hepatic ERα level is not rate-limiting in its role to maintain whole body lipid metabolism.

If the liver is not directly mediating the effects of estrogen on lipid metabolism, what could be the cause of the lipid changes seen after prolonged absence of the ERα or estrogen? Although we have not addressed this, it seems likely that secondary effects of estrogen signaling on other tissues that are involved in regulating lipid metabolism, such as brain, muscle and adipose tissue, play an important role in the development towards a change in lipid profile upon aging. In this respect, changes in for example adipose tissue distribution and size, as have been attributed to estrogen, would only have an effect on lipid metabolism beyond a certain level of change and thus time.

In conclusion, we find that short-term repression of hepatic ERα gene and protein expression does not have an overt impact on plasma and liver lipid levels. Apparently, the changes induced via the hepatic ERα are effectively compensated for or play a relatively minor role in maintaining cholesterol and triglyceride homeostasis.

Methods

Plasmids and Adenoviral vectors

The p.Empty, p.shERα plasmids and the Ad.Empty, Ad.ERE-Luc and Ad.shERα vectors have been generated as previously described [11].

Animals and Ad Injection

All animal work was approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands and the experimental protocols complied with the national guidelines for use of experimental animals. APOE*3-Leiden female mice were housed under standard conditions in conventional cages with free access to water and food. The study was performed in 17-19 weeks old APOE*3-Leiden mice (n=11) that were fed a Western type diet (Hope Farms, Woerden, The

Netherlands) starting 8 weeks prior to the experiment. For Ad-mediated gene transfer experiments, mice were transferred to filter-top cages, placed in a designated room, and allowed to adapt for at least five days. For in vivo adenoviral transductions, $2x10^9$ plaque forming units Ad.shERα or Ad.Empty in total volume of 200 µl (phosphate-buffered saline) were injected into the tail vein of mice. Within five days post-infusion, mice were sacrificed; liver pieces were removed and immediately deep-frozen in liquid nitrogen and stored at -80°C.

Plasma parameters

At day –6 and day 5 of Ad. injections, APOE*3-Leiden female mice were fasted for 4 h. Blood samples were taken via tail bleeding in paraoxon-coated capillaries, to prevent lipolysis [21]. Plasma was collected by centrifugation at 4°C. Plasma levels of total Chol and TG were determined enzymatically using commercially available kits and standards (Sigma Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany). Blood glucose levels were measured by a Freestyle hand glucose analyzer (Disetronic, Vianen, The Netherlands). All plasma parameters were determined according to the manufacturers' instructions.

Hepatic VLDL-TG production

At day 5 after $1.5.10^9$ pfu Ad.Empty or Ad.shERα administration, APOE*3-Leiden female mice were fasted for 4 h and then intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described [22]. Blood samples of Ad.Empty and Ad.shERα treated mice were collected 1, 30, 60, 90 and 120 min after Triton injection (n=4 and n=5 respectively). Serum TG concentrations were measured enzymatically, as described above. The hepatic VLDL-TG production rate was measured as the accumulation of serum TG after Triton injection and expressed as mg/dl/min.

Hepatic Lipid levels

Liver and muscle samples were homogenized in H_2O (~10% wet wt/vol). Lipids were extracted according to Blight and Dyer's method [23]. In short, a solution was made of each sample of 200 µg protein in 800 µl H_2O. 3 ml methanol/chloroform (2:1) was added and mixed thoroughly, after which 500 µl chloroform, 100 µl internal standard and 1 ml demi-water were added. After centrifugation the chloroform layer was collected and dried. The

remaining pellet was dissolved in 50 µl chloroform and put on a HPTLC plate. With HPTLC analysis, triglycerides, cholesterol and cholesterol esters were separated and the amount was quantified by scanning the plates with a Hewlett Packard Scanjet 4c and by integation of the density using Tina version 2.09 software (Raytest, Staubenhardt, Germany)

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 µg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [24]. PCR primer sets (table 2) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. HPRT was used as the standard housekeeping gene. The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)}$–$C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Table 2. Primer sequences of genes used for mRNA quantification

Gene	*Forward primer*	*Reverse primer*
HPRT	5'-TTGCTCGAGATGTCATGAAGGA	5'-AGCAGGTCAGCAAAGAACTTATAG
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
ApoE	5'-AGCCAATAGTGGAAGACATGCA	5'-GCAGGACAGGAGAAGGATACTCAT
IL-6	5'-AAGAATTTCTAAAAGTCACTTTGAGATCTA	5'-CACAGTGAGGAATGTCCACAAAC
ApoAV	5'-GAGCAAAGGCGTGATGGG	5'-TGCTCGAAGCTGCCTTTCA
SHP	5'-CTATTCTGTATGCACTTCTGAGCCC	5'-GGCAGTGGCTGTGAGATGC
Cyp7A	5'-CTGTCATACCACAAAGTCTTATGTCA	5'-ATGCTTCTGTGTCCAAATGCC
PPARα	5'-CCTCAGGGTACCACTACGGAGT	5'-GCCGAATAGTTCGCCGAAA

Western blot analysis

Immunoblotting procedures were performed as described previously [25]. Livers of both Ad.Empty and Ad.shERα treated mice (n= 5) were lysed and homogenized in 200 µl of RIPA

buffer (1% NP40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0, 150mM NaCl, 2,5mM EDTA) containing protease inhibitor (40ul/ml, Roche). Extracts were cleared by centrifugation (4°C, 14 000 *g*, 5 min), and protein content was determined using the BCA kit (Pierce). Protein samples were denaturated (5 min, 90°C) and separated on SDS/PAGE by use of 8% gradient gels and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). Blots were stained with Ponceau S before blocking to verify equal loading and appropriate protein transfer. Membranes were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween 20 and 10% milk powder. Thereafter, membranes were incubated for 16 h at 4°C with ab MC20, 1:1000 (mERα rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). After extensive washing with blocking buffer without milk powder or BSA, membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 (Promega). Membranes were again extensively washed and bound peroxidase conjugates were visualized by enhanced chemiluminescence (ECL, Amersham) on a LumiImager workstation. Additionally, filters were stripped by an 30 min incubation in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.8 at 50°C, to proceed with the whole procedure as described above. However, now membranes were incubated for 16 h at 4°C with p-38 ab, 1:1000 (N-20, cs-728, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). Immunoblots were quantified using Lumianalyst software on a LumiImager (Boehringer-Mannheim).

Statistical analysis

Results are presented as mean ± SD values for the number of animals indicated. Differences between the experimental groups were determined by Mann-Whitney U test. The level of statistical significance of the difference was set at $P < 0.05$.

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