RORα regulates the expression of genes involved in lipid homeostasis in skeletal muscle cells: Caveolin-3 and CPT-1 are direct target of ROR.

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Summary: The staggerer mice carry a deletion in the RORα gene, and have a prolonged humoral response, overproduce inflammatory cytokines, and are immunodeficient. Furthermore, the staggerer mice display lowered plasma ApoA-I/II, decreased plasma HDL cholesterol and triglycerides, and develop hypo-alphalipoproteinemia and atherosclerosis. However, relatively little is known about RORα in the context of target tissues, target genes, and lipid homeostasis. For example, RORα is abundantly expressed in skeletal muscle, a major mass peripheral tissue that accounts for ~40% of total body weight, and 50% of energy expenditure. This lean tissue is a primary site of glucose disposal, and fatty acid oxidation. Consequently, muscle has a significant role in insulin sensitivity, obesity and the blood-lipid profile. In particular, the role of RORα in skeletal muscle metabolism has not been investigated, and the contribution of skeletal muscle to the ROR -/- phenotype has not been resolved. We utilize ectopic dominant negative RORα expression in skeletal muscle cells to understand the regulatory role of RORs in this major mass peripheral tissue. Exogenous dominant negative RORα expression in skeletal muscle cells represses the endogenous levels of RORα and γ mRNAs, and ROR dependent gene expression. Moreover, we observed attenuated expression of many genes involved in lipid homeostasis. Furthermore we show that the muscle carnitine palmitoyl-transferase-1, and Caveolin-3 promoters are directly regulated by ROR and co-activated by p300, and PGC-1. This study implicates RORs in the control of lipid homeostasis in skeletal muscle. In conclusion, we speculate that ROR agonists would increase fatty acid catabolism in muscle, and suggest selective activators of ROR may have therapeutic utility in the treatment of obesity, and atherosclerosis.
INTRODUCTION

Members of the nuclear hormone receptor (NR) superfamily bind specific DNA elements and function as transcriptional regulators (1,2). This group includes the 'orphan NRs' which have no known ligands in the 'classical sense'. The orphan receptor, ROR/RZR (retinoic acid receptor related orphan receptor), is closely related to Rev-erbAα, RVR/Rev-erbβ/BD73 and the Drosophila orphan receptor, E75A, particularly in the DNA-binding domain (DBD) and the putative ligand-binding domain (LBD). ROR, Rev-erbAα, and RVR bind as monomers to an asymmetric (A/T)₆RGGTCA motif. ROR functions as a constitutive trans-activator of gene expression, in contrast, Rev-erbAα and RVR, do not activate transcription, mediate transcriptional repression, and can repress constitutive trans-activation from this motif by RORα (3-9).

Three ROR/RZR genes have been identified; RORα encodes four RORα isoforms α1, α2, α3 and RZRα, which are alternatively spliced products of the RORα gene and are predominantly expressed in blood, brain, skeletal muscle and fat cells (8,10). RORβ/RZRβ is expressed specifically in the brain (11) and RORγ is found at high levels in skeletal muscle (12-14).

Genetic studies have implicated RORα in the regulation of lipid homeostasis. For example analysis of RORα function has been assisted by a natural mutation in an obese mouse strain called staggerer (sg/sg). The mutation produces a dominant negative RORα that results in a functional knockout phenotype. In accordance with the wide expression pattern of RORα, staggerer mice show symptoms of ataxia and cerebellar dysfunction, susceptibility to atherosclerosis and hypoalphalipoproteinemia, vascular dysfunction, muscular irregularities, osteoporosis, and severe immune abnormalities. In the context of lipid homeostasis the staggerer mice exhibit an aberrant blood lipid profile with lower circulating plasma levels of HDL-C, Apolipoprotein (Apo) CIII and plasma triglycerides.
Decreases in specific lipoprotein compartments namely ApoAI, the major constituent of HDL, and ApoAII leads to a pronounced hypoalphalipoproteinemia (16). Accordingly, it has been shown that ROR\(\alpha\) regulates the expression of ApoAI and ApoCIII in cell culture (16,17). Susceptibility to atherosclerosis in this animal model is linked to a complex phenotype that includes aberrant vascular physiology, lipid profiles and hyper-sensitive inflammatory responses.

Studies examining motor capabilities and coordination in \textit{staggerer} mice have shown a reduction of muscular strength in these mice (18). Furthermore, the studies of Lau et. al. (19) demonstrated that ROR\(\alpha\) potentiates skeletal muscle myogenesis and the comments in a review by Jarvis et. al. (20) suggest that staggerer mice have skeletal muscle irregularities.

The association between ROR\(\alpha\), lipid homeostasis and skeletal muscle is not surprising. ROR\(\alpha\) is abundantly expressed in skeletal muscle, which is one of the most metabolically demanding tissues that relies heavily on fatty acids as an energy source, and accounts for 75% of glucose disposal. Moreover, this suggest that muscle has an important role in obesity, which is primarily controlled by increased food intake or decreased energy expenditure. However, the fundamental role of ROR\(\alpha\) in skeletal muscle cholesterol, lipid, glucose and energy homeostasis has not been examined. Moreover, the contribution of skeletal muscle, a major mass tissue, to the ROR knockout phenotype has not been resolved.

The involvement of ROR\(\alpha\) in the progression of atherosclerosis, identifies ROR\(\alpha\) as a therapeutic target in the treatment of cardiovascular disease. In addition, skeletal muscle is rapidly emerging as a critical target tissue in the battle against obesity, type II diabetes,
dyslipidemia, syndrome X and atherosclerosis. NRs in skeletal muscle, for example LXR, PPARα, β/δ and γ, have been shown to be involved in enhancing insulin stimulated glucose disposal rate, decreasing triglycerides and increasing lipid catabolism, cholesterol efflux and plasma HDL-C levels (21-23). Hence, orphan NRs that regulate lipid and glucose metabolism, cholesterol homeostasis, energy expenditure and thermogenesis in skeletal muscle have enormous pharmacological utility for the treatment of dyslipidemia, and syndrome X.

We hypothesize that RORα regulates lipid, glucose and energy homeostasis in this major mass lean tissue. This hypothesis was addressed by examining the effect of ectopically expressing the staggerer form of RORα in muscle cells, and investigating the subsequent effect on gene expression involved in lipid metabolism. Our investigation reveals that ROR has a central role in the regulation of lipid metabolism in muscle cells. Moreover, we suggest that the atherogenic and dyslipidemic phenotype of the staggerer mouse is related to aberrant RORα function in skeletal muscle.
MATERIALS AND METHODS:

Cell Culture: Mouse myogenic C2C12 cells were cultured in growth medium [DMEM supplemented with 10% Serum Supreme (BioWhittaker, Edward Keller Pty Ltd, Hallam, Victoria, Australia)] in 6% CO2. Myoblasts were differentiated into post-mitotic multinucleated myotubes by 1-5 days of serum withdrawal (i.e. cultured in DMEM supplemented with 2% horse serum). Cells were harvested at indicated time points, usually 120h (5 days) after the mitogen withdrawal, unless indicated differently. African green monkey kidney COS-1 cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum.

Transient Transfections: Each well of a 24 well plate of COS-1 cells (~60% confluence) was transfected with a total of ~ 0.6-0.8 μg DNA per well) using the liposome mediated transfection procedure as described previously. Cells were transfected using a DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) and Metafectene (Biontex Laboratories GmbH, Munich, Germany) liposome mixture in 1 x HBS [HEPES buffered saline (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na2HPO4, 11 mM dextrose (pH 7.1)]. The DNA/DOTAP/Metafectene mixture was added to the cells in 0.5-0.6 mls of DMEM supplemented with 5% charcoal stripped fetal calf serum. 16-24h later, the culture medium was changed, and the cells were subsequently harvested for the assay of Luciferase activity 24-48h after the transfection period as described previously. Fold activation is expressed relative to LUC activity obtained after cotransfection of the reporter, and pSG5 vector only, arbitrarily set at 1. The mean fold activation values and standard deviations (bars) were derived from a minimum of 2 independent experiments comprised of six replicates.

CAT assays were previously described (Lau et al, 1998). Transient transfections were performed at least twice in sextuplicate.

C2C12 stable transfection: Myogenic C2C12 cells cultured in growth medium, were co-transfected with pSG5-RORΔDE (and pCMV-NEO at a 20:1 ration of expression vector:NEO
vector) by the liposome-mediated procedure in duplicate. The cells were then grown for another 24 h to allow cell recovery and neomycin resistance expression before G418 selection. After 10-14 days selection with 450 µg/ml G418 (Invitrogen) in culture medium, the two independent polyclonal pools (>100 colonies per pool) of stable transfectants were cultured and maintained on 200 µg/ml G418 medium.

**RNA Extraction**: Total RNA was extracted from C2C12 using TRI-Reagent (Sigma Aldrich Australia Pty Ltd, Castle Hill, New South Wales, Australia), according to manufacturer’s protocol. RNA for Quantitative Real-Time (Q-RT)-PCR, was further purified using RNeasy (QIAGEN, Clifton Hill, Victoria, Australia), according to manufacturer’s instructions. This was followed by DNase treatment with DNA-free (Ambion, Austin Tx.)

**Quantitative Real Time (Q-RT)-PCR**: RNA was normalized using UV spectrophotometry and agarose gel electrophoresis. Complementary DNA was synthesized from 7ug of total RNA by Superscript3, as according to manufacturer’s instruction (Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia). Target cDNA levels were analyzed by Q-RT PCR in 25ul reactions containing either 1x SYBR green (ABI, Warrington, UK) or Taqman PCR master mix (ABI, Branchburg, NJ), 200nM each forward and reverse primers or Assay on Demand Taqman primers (ABI, Foster City CA) and cDNA (0.6% of the starting 7ug RNA) using an ABI Prism 7000 Sequence Detector system. PCR was conducted over 45 cycles of 95 C for 15 sec and 60 C for 1 min two-step thermal cycling preceded by an initial 95 C for 10 min for activation of Amplitaq Gold DNA polymerase.

**Primers**: *Mus musculus* primer sequences (forward and reverse, respectively):

- **ABCA1**: GCTCTCAGGTGGGATGCAG, GGCTCGTCCAGAATGACAAC;
- **18S**: GATCCATTGGAGGGCAAGTCT, CCAAGATCCAACTACGAGCTTTTTT
- **A8/G1**: CTGAGGGATCCTGGGTCTGA, CCTGATGCCACTTCCATGA;
- **ACS4**: GGTTTGGTAACAGATGCCTTCAA, CCCATACATTCGCTCAATGTCTT;
ADRP: CCCTGGTTCTAAGAAGCTGCTTT, GGCCAGATGACCCCTTTTG;
AdipoR1: ACGTTGGAGAGTCATCCCGTAT, CTCTGTGTGGATGCGGAAGAT;
AdipoR2: TCCCAGGAAGATGAAGGGTTTAT, TTCCATTCGTTCGATAGCATGA;
ApoE: GCTGTTGGTCACATTGCTGA, TGCCACTCGAGCTGATCTG;
β-actin: GCTCTGGCTCCTAGCACCAT, CCACCGATCCACACAGAGTAC;
CD36: GGCCAAGCTATTGCGACAT, CAGATCCGAACACAGCGTAGA;
CAV3: CTGCCACCCAGGACTATCAAC, TTCCAGATCCGTGTGCTCTTC;
CPT1b: ATCATGTATCGCCGCAAACT, CCATCTGGTAGGACACATGG;
FABP3: CCCCTCAGCTCAGCACCACCAT, CAGAAAAATCCCAACCAAGAAT;
FAS: CGGAAAGCTTTCAAGAAATGTC, TCAGAGACGTGTCAGTCCTCTGG;
GAPDH: GTGTCCGTCGTGGATCTGA, CCTGCTTCACCACCTTCTTG;
Glut4: ATGGCTGTCGCTGGATCTGA, CCTGCTTCACCACCTTCTTG;
Glut5: CTTGCCTTTACCGGGTTTGAC, CATCTGGTCTTGCAGCAACTCT;
MyoG: CTTAAAGCAGAGAGCATCC, GGAATTCCAGGGCATATAATAGA;
LPL: CCAATGGAGGCACTTTCCA, TGGTCCACGTCTCCGAGTC;
mRORα: CAATGCCACCTACTCTCTGTC, GCCAGGCATTCTTCTGCAGC;
PGC-1: CGATGTGTCGCTTCTTCTG, CGAGAGGCAGCTCCTTTCTG;
Rev-erb: CCACACCGCTGGGAGAGT, GCCCTGGGCTAGACCCCTCT;
RVR: GGAAACACTCATCCCGTGCACAA, ATCCGAAGATCTGGGCAACTTATG;
SCD1: TGTACGGGATCACTGTTGAC, ATCGAAGATCTGGGCAACTTATG;
SCD2: ACTGTGACTCAAGTTCAACTCTTG, TGCCCACAAATTGAGGATAGC;
SREBP-1c: CGTCTGCACGCCCTAGG, CTGGAGCATGTCTTCAAATGTG;
TNNI1: GCCTATGCGCACACCTTTG, CGGGTACCATAAGCCCACACT;
TNNI2: AAATGTTCGAGTCTGATCTCTAATTG, GCCGAAGTACTCCAGACTGGAT;
UCP-2: GCTCTTCTGTCTGCTTTCG, GGGCTTGAAACCAACCACCT;
UCP-3: TGACCTGCGCCACAC, CCCAGGCGTATCATGCT.
Human RORα specific primers contain 3 out of 5 base difference at the 3’ end of the reverse primer relative to mouse primers. hRORα: CAATGCCACCTACTCCTGTCC, CTACGGCAAGGCATTTCTGTAAT. GAPDH and 18S were used for normalization between samples for quantitation. Assay on Demand primers were used for Taqman real time PCR (ABI, Foster City CA) m+hROR (Mm00443103_m1); RORγ(Mm00441139_m1); FABP4 (Mm00445880_m1); GAPDH (Mm99999915_g1); 18S rRNA (Hs99999901_s1).

**Plasmids:** Human pSG5-RORα1 has been previously described (19). RORE x5-TKLUC contains 5 copies of RORE (TATATCAAGGTCAT) was cloned into pTKLuc as described according to Medvedev et. al. (24). mPCP-2 x4-tkLUC contain 4 copies of mouse Pcp-2 RORE (GTTATAGTAACTGGGTCAGGGGACT) was cloned into pTKLuc as described by Matsui (25). The inserted sequences in the TKLuc clones were confirmed by big dye terminator V3.1 cycle sequencing (ABI, Foster City, CA). The CPT1 promoter (-1025/-12) was kindly provided by D. P. Kelly (26) cDNA-GRIP1/SRC2 and cDNA-p300 have been described previously (27). CDNA4-PGC-1 was kindly provided by B. M. Spiegelman (28). 2.6 kb mouse caveolin-3 promoter was isolated from a BAC clone 23N20 (Incyte Genetics) and cloned into HindIII site of pGL3basic. Deletion constructs of the Cav3 promoter were obtained using native restriction sites or by PCR with pfu DNA polymerase. The DNA sequences of these constructs were confirmed by cycle sequencing (ABI). GLUT4-pGL2 was obtained by PCR cloning of a mouse skeletal muscle genomic DNA into pGL2basic. The DNA sequence contains 2.2kb sequence upstream of the transcription start site was confirmed by cycle sequencing. pGEX-RORα1 were previously described (19); GAL4-ROR constructs were cloned in-frame into the multiple cloning site of vector pGAL0 and confirmed by dideoxy sequencing (Pharmacia Uppsala, Sweden).
**Site directed mutagenesis:** pGL3-Cav3Luc (-2595/+35) was mutated by quickchange PCR site directed mutagenesis (Stratagene) according to manufacturer instruction. 125ng oligonucleotides containing GG to TT mutation and its complementary strand (see sequences below) were added to a PCR reaction containing 50ng pGL3-Cav3Luc (-2595/+35), dNTP, 1x pfu buffer and pfu or pfu ultra. After restriction digestion with dpnI, the reaction was transformed into DH5α. The colonies were screened and sequence confirmed by cycle sequencing after DNA extraction. The oligonucleotides to mutate the mouse caveolin 3 promoter were: Cav3 mut2 oligos

GAGGATACAATCTCGTGTCAAAATACTCACCCCGCCCCG,

CGGGGCGGGTGAGTATTTGACACGAGATTGTATCTCT; and the Cav3 mut6 oligos CATGCGGCTCCACGAGTAAAAGACTTCCCTCTCTCGAG,

CTCGAGAGGAGGAAGTCTTTCACTGGGGAGCCGCATG.

**Oligonucleotide probes and EMSA:**

Double-stranded oligonucleotide probes used in EMSA and for cold competition were:

RORE, 5'-TCGACTCGATATATCAAGGTCAAGCGTG-3' (Giguere et al 1994);

Mouse pcp-2, 5'-GTTATAGTAACTGGGTCAGGGGACTC-3' (Matsui, 1997);

Cav3 m1  CTGAGAACTACGTACATGGCCCTTTG
Cav3 m2  CTCGTGTCACCTACTCACCAG
Cav3 m3  CTGCTCCAAGGCACAGGAGAGCAGAGC
Cav3 m4  AGCAGAGCAAGGTCTTTGTCCCTTGGTGTC
Cav3 m5  CATGGACAAGGTAAAGGGGAGGCTTATAACTG
Cav3 m6  CCAGTGACCGACTTCCTCCTCTC
GST-RORα1 was prepared as previously described (Lau et al 1998 and Dowhan et al 1994). 100ng of double stranded oligonucleotide was radiolabeled with 5ul 32P γ-ATP and T4 PNK and purified by 6% TBE-PAGE.

EMSA were carried out as previously described with modification (Dowhan et al 1994 and Curlewis et al 2002). 0.4 ug GST-RORα1 protein in Dignam buffer C (0.1M NaCl, 20mM HEPES pH7.9, 2mM EDTA, 1mM DTT and 1x complete protease inhibitor (Roche)), was incubated with 1ug poly dIdC.dIdC, 4ug BSA, 1x complete protease inhibitor (Roche), 300cps of gel purified 32P double stranded radioactive probe end labeled by T4 PNK, 12% glycerol, 0.6mM DTT and 1x binding buffer (Preitner et al, 2002) on ice for 30 min before gel separation. Cold competition studies were carried out by co-incubation with 100-300ng of double stranded unlabeled probe.
RESULTS:

ROR α and γ mRNA expression are induced during myogenic differentiation

RORα and γ mRNAs are expressed in skeletal muscle tissue. To elucidate the functional role of RORα in skeletal muscle we initially investigated the expression of RORα mRNA relative to the 18S rRNA in the mouse C2C12 myoblast cell line. This skeletal muscle cell line has proven to be a reliable system to study skeletal muscle lipid homeostasis in cell culture (21-23).

Proliferating C2C12 myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by serum withdrawal in culture over a 48-96 h period. This transition from a non-muscle phenotype to a contractile phenotype is associated with the repression of non-muscle proteins and the activation/expression of a structurally diverse group of genes responsible for contraction and to cope with the extreme metabolic demands on this organ (Figure 1A).

Using the Genbank sequences of ROR α and γ we designed specific primers for the amplification of mouse ROR by quantitative real time PCR from total RNA isolated from proliferating and differentiated C2C12 cells (Figure 1A). Total polyA+ RNA was isolated from proliferating myoblasts (PMB), confluent myoblasts (CMB) and post-mitotic myotubes after 2 and 5 days (MT2 & MT5) of serum withdrawal and examined by real time PCR and 'Northern Blot' analysis.

We observed that RORα and γ are expressed in proliferating myoblasts, however, these transcripts are induced 2.5-4 fold, relative to 18S rRNA and GAPDH mRNA (data not shown), as the cells exit the cell cycle and fuse to form differentiated multinucleated myotubes that have acquired a muscle-specific phenotype (Fig. 1B and D). RORα is seen transcribed as a double band (Figure 1C) in C2C12 as described in differentiated P19 cells (3). Concomitant with this increase in ROR encoding mRNAs, was the striking induction of
myogenin mRNA (figure 1E), that encodes the hierarchical bHLH regulator, and repression of the cytoskeletal non-muscle β-actin mRNA (Figure 1F), that confirmed that these cells had terminally differentiated. Moreover, we observed the induction of the slow and fast isoforms of the contractile protein, Troponin I (Figure 1G and H). These data demonstrated that the RORα and γ mRNA transcripts were expressed in C2C12 cells and expression is increased upon myotube formation.

Induction of RORα and γ mRNAs correlated with the repression of the mRNAs encoding the closely related but opposingly acting Rev-erbα and β(RVR) orphan nuclear receptors (Figures 1I and J) which are abundantly expressed in proliferating myogenic cells, but are repressed during myogenic differentiation. This is in concordance with previous observations (50-51).

Ectopic RORΔE expression represses endogenous levels of ROR α and γ, and attenuates ROR dependent gene expression.

To understand the biological role of RORα in skeletal muscle lipid homeostasis and to identify the metabolic target(s) of this orphan receptor in muscle cells, we proceeded to examine the effect of attenuating ROR function in C2C12 cells.

In order to perturb ROR function, and disrupt ROR mediated gene expression we utilized the RORΔE plasmid. This construct encodes amino acids 1-235, but lacks the entire E region and part of the hinge/D-region of RORα. This was chosen because McBroom et al (30) reported that deletion of this region preserved DNA binding but destroyed trans-activation, and operated in a dominant negative manner. Furthermore, the 'staggerer' phenotype in mice is due to a frameshift mutation in RORα (29), that produces a similar non-functional C-terminal domain. Finally, we noted that GAL4 hybrid analysis
indicated that the RORα activation domain was located downstream of aa position 235 (Figure 2A). Moreover, in concordance with this observation we demonstrated that RORΔDE (RORα1 1-235) ablated the ROR mediated trans-activation of the synthetic (RORE x 5) and native (mPCP-2x4) ROR-dependent response elements linked to the basal tk-LUC reporter (Figure 2B and C). Also shown, is the opposingly acting RVR/rev-erb β orphan which does not trans-activate the RORE’s. Moreover, the RORΔDE repressed the ROR-mediated trans-activation of the synthetic heterologous RORE-tk-LUC reporter in a dose dependent manner, similar to the orphan receptor, RVR (Figures 2 D and E).

This demonstrated that this construct as expected from studies on the staggerer form of ROR, and other forms of ROR that lack the entire E region and parts of the hinge, attenuated ROR dependent gene expression.

We stably (and independently) co-transfected two populations of C2C12 cells with RORΔDE and pCMV-NEO expression vectors. Stable RORΔDE transfectants were isolated as two independent polyclonal pools of G418-resistant colonies (comprised of >20 individually resistant colonies). The C2:RORΔDE cells were transfected with pSG5-RORΔDE (i.e amino acids 1-235). The two polyclonal pools of C2-RORα1 (1-235) cells abundantly expressed the ectopic/exogenous human transcript relative to GAPDH and 18S rRNA controls. This was demonstrated rigorously by quantitative real time PCR with primers that specifically detect the human specific transcript expressed from the expression vector (Figure 3A and B) and northern analysis (Figure 3C). Furthermore, the total pool size of RORα mRNA increased ~2-3 fold when measured by real time PCR with primers that detected both mouse and human transcripts (Figure 3D). Interestingly, we observed by real time PCR and northern analysis that the endogenous levels of the mouse RORα mRNA transcripts were reduced in both polyclonal pools of the C2-RORα1 (1-235) cells (Figure 3E and F) relative to the wild type levels. The down regulation of the endogenous RORα
transcripts is not surprising. During myogenesis mRNA pool sizes in muscle tissue are under strict control (31), mechanisms exist that sense total output from exogenous and endogenous genes (32). Furthermore, exogenous expression of a number of different contractile protein transgenes in the mouse (eg myosin light chain 2, troponin I fast, skeletal and cardiac actin) results in the decline in the expression of the corresponding endogenous gene (32-36). Finally, we observed that ectopic expression of the RORαDE in muscle C2 cells completely ablated RORγ mRNA expression (Figure 3G).

In conclusion, the cell lines ectopically expresses a dominant negative RORα expression vector, that compromises ROR function, and ROR dependent gene expression. Moreover, RORα mRNA expression is attenuated, and RORγ mRNA expression is ablated.

**ROR regulates lipid homeostasis: Dominant negative ROR activity represses the expression of genes involved in lipid absorption, utilization and efflux.**

We investigated the expression of the genes involved in skeletal muscle lipid and carbohydrate metabolism (see table 1) in the wild type and the RORΔDE expressing C2C12 cells. This *in vitro* cell culture system has been used to investigate the regulation of cholesterol homeostasis and lipid metabolism by liver X receptor (LXR) agonists (22). Muscat *et al.* demonstrated that the selective and synthetic liver X receptor (LXR) agonist, T0901317 induced similar effects on mRNAs encoding ATP binding cassette A1/G1 (ABCA1/G1), apolipoprotein E (ApoE), stearoyl CoA desaturase-1 (SCD-1), sterol regulatory element binding protein-1c (SREBP-1c), etc. in differentiated C2C12-myotubes and *Mus musculus* quadriceps skeletal muscle-tissue. Moreover, treatment of these cells with peroxisome proliferators activated receptors (PPAR) α and δ agonists and an analysis of
critical genes (>12) involved in lipid and carbohydrate metabolism revealed similar responses to quadriceps tissue from agonist treated C57BL/6J mice (21-23). The physiological validation of the cell culture model with respect to lipid homoestasis in the mouse corroborates the utility of this model system. This evidence, coupled to the flexibility and utility of cell culture in terms of manipulation, RNA extraction and target validation provides an ideal platform to identify the RORα-dependent regulation of genes involved in metabolism. In addition, and more importantly we have demonstrated this cell line expresses RORα, and -γ receptors, and that the stable cell line produced by ectopic RORA∆E over-expression has compromised endogenous ROR α/γ expression, and function.

We isolated total RNA from the native (wild type) and RORA∆E differentiated myotubes, and analysed the expression levels of several myogenic mRNAs by quantitative real time PCR. We demonstrated as previously reported that these cells retain the potential to differentiate (although the initial rate is impaired slightly), exit the cell cycle, and express all genes associated with contraction, and lipid metabolism (data not shown). This suggests that ectopic RORA∆E expression does not significantly affect proliferation, cell cycle withdrawal, differentiation and/or phenotypic acquisition of these skeletal muscle cells.

Subsequently, we utilized quantitative real-time PCR to investigate the expression pattern of genes involved in fatty acid and lipid absorption (fatty acid translocase (FAT/CD36), fatty acid binding protein 3 & 4 (FABP3 & 4), triglyceride (TG) hydrolysis (lipoprotein lipase (LPL)), and fatty acid oxidation (muscle type carnitine palmitoyltransferase-1 (M-CPT1), acyl-CoA synthetase-4 (ACS-4), adiponectin receptors 1 and 2), glucose/fructose absorption and utilisation (glucose transporter-4, & -5 (GLUT-4 & -5), in total RNA isolated from C2C12 and C2:RORA∆E myotubes differentiated for 5 days.
In the context of lipid and fatty acid uptake we observed significant repression of the mRNAs encoding fatty acid translocase (FAT/CD36), and fatty acid binding protein 3 (FABP-3), with only minor changes observed for fatty acid binding protein 4 (FABP-4) in the RORΔE expressing cell line, respectively (Figure 4A-C).

The transcripts encoding lipoprotein lipase, muscle type carnitine palmitoyltransferase-1 (M-CPT1), and acyl-CoA synthetase-4 (ACS-4) which are involved in triglyceride-hydrolysis, fatty acid-oxidation and preferential fuel utilization were significantly repressed ~5-7-fold in the RNA isolated from the cell line over-expressing RORΔE (Fig. 4F-H). The significance of the repression is highlighted by the observation that expression of peroxisome proliferators activated receptor-gamma coactivator-1 (PGC-1) mRNA (Figure 4K), myogenin (myoG) mRNA (Figure 4W) [that encodes the hierarchical bHLH regulator], and β-actin mRNA(Figure 4X) were reduced ~2-fold. Moreover, the changes observed are not due to the effects of RORΔE expression on differentiation. Over a five day period the cell line retained the potential to differentiate as measured by the induction and activation of myoG, (Figure 4Y), troponin I slow [(TNNI1) Figure 4A(ii)], troponin I fast [(TNNI2) (Figure 4B(ii)], and repression of β-actin (Figure 4Z) mRNA expression, respectively after serum withdrawal.

Interestingly, the adiponectin receptors 1 and 2 that mediate increased AMP kinase levels, as well as fatty acid oxidation and glucose uptake by adiponectin were significantly repressed (Figures 4I and J). This correlates with the significant repression of glucose transporter 4 (GLUT-4) mRNA expression but not glucose transporter 5 (GLUT-5) mRNA
(Figure 4D and E). However, we note that these cell lines express very low levels of the glucose transporters 4/5 (GLUT-4/-5) transcripts.

Subsequently, we examined the expression of genes involved in fatty acid and lipid absorption, lipid and cholesterol efflux (ATP binding cassette A1 & G1 (ABCA1/G1), apolipoprotein E (ApoE)), lipid storage and homoeostasis (adipophilin/adipocyte differentiation-related protein (ADRP), and caveolin-3 (CAV3)); lipogenesis (sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS), stearoyl CoA desaturase-1 & -2 (SCD-1 & -2); Fig. 4B); Fig. 4E), and energy expenditure (uncoupling protein-1, -2, & -3 (UCP-1,-2 &-3)).

In the two polyclonal populations of cells expressing RORΔE dramatic repression we observed dramatic reduction in the expression of mRNAs encoding ATP binding cassette A1 (ABCA1) and apolipoprotein E (apoE), but minor/subtle changes in ATP binding cassette A8/G1 (ABCA1/G1) levels (Figures 4N, O and P). Interestingly, we observed significantly decreased levels of the mRNA encoding caveolin-3 (CAV3) (Figure 4Q), the major structural protein of caveolae in skeletal muscle, which has been implicated in cholesterol homeostasis. Similarly, we observed reduced expression of adipocyte differentiation-related protein (ADRP) mRNA, a protein involved in lipid droplet formation. (Figure 4R).

The mRNAs encoding the hierarchical regulator of lipogenesis, sterol regulatory element binding protein-1c (SREBP-1c) (Figure 4S) and the lipogenic genes (stearoyl CoA desaturase-1 & -2 (SCD-1 & -2) and fatty acid synthase (FAS))(Figure 4T, U and V) were all notably reduced in the RORΔE expressing cell lines.
Furthermore, the mRNAs encoding uncoupling protein-2 and –3 (UCP-2 and –3) which encode uncoupling proteins involved in energy uncoupling/expenditure and preferential lipid utilization, were significantly repressed by the expression of ectopic dominant negative RORα (Figures 4 L and M).

Finally, the expression of the closely related, but opposingly acting orphan receptors, Rev-erβα and Rev-erββ/RVR was further repressed by RORΔE expression (Figures 5A and B). This was expected as RORα has been demonstrated to regulate the Rev-erβα gene.

PGC-1 efficiently coactivates the synthetic and native RORE heterologous reporter genes.

We then investigated the ability of ROR to activate the synthetic and wild type-ROR dependent reporter in COS-1 cells, and the ability of the cofactors p300, SRC-2/GRIP-1, and PGC1 to coactivate ROR dependent activation of gene expression. We utilised the synthetic ROREx5-tk-LUC reporter that contains five copies of a consensus binding site (24), and the native mPCP-2x4-tk-LUC containing four copies of the mouse purkinje cell protein-2 RORE motif (25) cloned upstream of the heterologous herpes simplex virus thymidine kinase (tk) promoter linked to the luciferase reporter gene, respectively. As shown RORalpha expression efficiently activated the expression of the synthetic (Figure 6A) and native RORE (Figure 6B)-containing reporters in COS-1 cells, several hundred fold. In contrast, RORα relatively insignificantly (i.e. <10-fold) regulated the basal tk-LUC-backbone, lacking the RORE (Figure 6C).
Furthermore, we observed that RORα dependent activation of the synthetic RORE was significantly enhanced by the coactivator, PGC1, relative to p300 and SRC-2/GRIP-1. Similarly, ROR dependent activation of the native RORE was most efficiently coactivated by PGC-1, relative to p300 and SRC-2/GRIP-1. In summary, we demonstrate that PGC-1 expression selectively coactivates RORα mediated activation of the heterologous synthetic and native RORE-tk-LUC reporters.

M-CPT1 And Cav-3 Genes Are Primary Targets Of RORα In Skeletal Muscle.

We further explored the molecular basis of ROR mediated gene activation in skeletal muscle cells by evaluating whether direct, or indirect mechanisms mediated the observed changes in mRNA-levels in the RORΔDE cells. We investigated the responsiveness of the promoters of selected target genes to RORα co-expression in a cell-based reporter assay.

We transiently transfected COS-1 cells with the regulatory sequences of selected target genes, including ABCA1 (22), CD36/FAT (37), LPL (38), M-CPT1 (26), CAV-3, GLUT4 and UCP-2 (39), cloned in front of the pGL2/3-basic luciferase backbone and examined the response after cotransfection of RORα in the presence and absence of p300, SRC-2/GRIP-1 and PGC-1.

Interestingly, the M-CPT1-promoter (and not several other promoters including GLUT4) which contain 1025 bp upstream of the start of translation was efficiently activated by ROR expression (Figure 7A). Moreover, we observed that p300 and PGC1 significantly enhanced the transactivation of the M-CPT1 (Figure 7B). The transcriptional coactivators p300 and PGC-1 are expressed in skeletal muscle and have been demonstrated to potentiate
myogenesis, and mitochondrial biogenesis, oxidative metabolism, fiber type transitions [fast (type II) to slow (type I)] and thermogenesis. Furthermore, we observed similar ROR dependent activation, and cofactor preference with a VP16-RORα chimera. (Figure 7C).

Interestingly, the p-mCav3-2595-LUC promoter that encompasses 2595 bp immediately upstream of the murine Cav-3 transcription start site (that drives the expression of the muscle-specific caveolin-3 gene) was also efficiently activated by RORα expression (Figure 8A).

In an attempt to further elucidate which sequences were necessary for the trans-activation of the mCav-3-LUC promoter by RORα, we utilized a series of 5' unidirectional deletions in the mCav-3 promoter. Several deletion constructs were produced, namely, p-mCav3-988-LUC, p-mCav3-654-LUC, p-mCav3-477-LUC, and p-mCav3-279-LUC which contained 988, 654, 477 and 279 nucleotides, respectively, of 5' flanking sequences cloned upstream of the luciferase reporter in pGL2-Basic. By transfecting the full-length and deleted promoters in the absence/presence of the RORα expression vector in COS-1 cells, we could demonstrate that the sequences between nt position –2595 and –988 were necessary for RORα dependent activation (Figure 8A). Moreover, we observed that p300 and SRC-2/GRIP-1 significantly enhanced the transactivation of the mCav-3 promoter. (Figure 8B).

To rigorously define the molecular basis of direct ROR action, we focused on one of the promoters that we defined as a primary ROR target gene. We selected and scanned the mouse caveolin-3 promoter by the Mat-inspector program to identify putative AGGTCA nuclear receptor binding motifs. We observed six putative ROR monomeric binding motifs m1-m6, located between nt positions –2498/-2474, 2378/-2357, -1739/-1713, -1720/-1690, -1587/-1556, and –1005/-984, respectively (Figure 8C). These motifs to varying degrees could...
be accommodate by the asymmetric (A/T)$_6$RGGTCA ROR recognition motif, and were located in the delimited region between nt positions –2595 and –988 that were necessary for ROR$\alpha$ dependent activation.

Subsequently, we examined the ability of the motifs 1-6 to interact with GST-ROR, and observed that motifs 2, 4, 5 and 6 bound ROR with different efficiencies in an EMSA assay, relative to our control ROR recognition motif, the mouse PCP-2 RORE (Figure 8D). However, we observed that only the m2, and m6, (and the synthetic RORE), but not the m1, M3-M5 motifs) efficiently competed against the efficient GST-ROR-mPCP-2 RORE interaction (Figure 8E). This suggested that either motif 2 or 6 mediated ROR activation of the mouse caveolin-3 gene. Hence, we independently mutated the GG nt in the nuclear receptor halfsites to TT in the m2 and m6 putative RORE’s within the native caveolin-3 promoter, and examined the ability of ROR (in the presence of the cofactor p300) to trans-activate the caveolin-3 promoter. We observed that the mutation of motif 6 in the mouse caveolin-3 promoter compromised the ability of ROR to trans-activate the promoter (Figure 8F). This was also consistent with the analysis of several additional 5’ unidirectional deletion mutant of the caveolin-3 promoter between –2595 and –984 that were examined (data not shown).

In summary, these transfections demonstrated that ROR$\alpha$ directly regulates the CPT-1 and Cav3 genes that potentially encode proteins involved in beta-oxidation and cholesterol homeostasis. Moreover, we defined the specific sequences in a muscle-specific gene (i.e. caveolin-3) that mediated the direct regulation of expression by ROR. This suggests that ROR$\alpha$ has a central role in the regulation of lipid homeostasis.
DISCUSSION:

Genetic, molecular and biochemical studies have demonstrated that NR1F1 (RORα) deficient mice develop severe atherosclerosis, low HDL-C, hypoalphalipoproteinemia, muscular atrophy and heightened inflammatory responses. Moreover, NR1F1 regulates the expression of Apolipoprotein CIII, a component of HDL and VLDL that plays a role in regulation of triglyceride levels and lipoprotein lipase activity. Furthermore, RORα potentiates the acquisition of the skeletal muscle phenotype.

The expression of RORα in skeletal muscle, a peripheral lean tissue that has a significant role in energy consumption, glucose disposal and free fatty acid uptake prompted us to investigate the regulatory role of RORα in skeletal muscle cells, with respect to gene expression involved in metabolism. Our study demonstrates that RORα regulates the expression of genes involved in lipid absorption, β-oxidation, cholesterol efflux, and energy expenditure in skeletal muscle cells. The observed changes in gene expression in skeletal muscle are consistent with the phenotype in this animal model.

The natural RORα mouse mutant, called staggerer (sg/sg) is susceptible to high fat diet induced atherosclerosis, with atherosclerotic lesions in the aorta, and the large and small coronary arteries. In the context of this investigation, the staggerer mice exhibit an aberrant blood lipid profile with lower circulating plasma levels of HDL-C (associated with decreased plasma apoA-I and apoA-II), reduced Apolipoprotein (Apo) CIII and plasma triglycerides. Decreases in specific lipoprotein compartments namely ApoAI, the major constituent of HDL, and ApoAII leads to a pronounced hypoalphalipoproteinemia (16). Accordingly, it has been shown that RORα regulates the expression of ApoAI and ApoCIII in cell culture (16,17). Further studies in staggerer mice, correlate atherogenic susceptibility in this animal
model to a complex phenotype that includes aberrant vascular physiology, lipid profiles and inflammatory responses. However, the contribution of skeletal muscle (atrophied in the RORα sg/sg mice), and other putative target tissues to the staggerer phenotype has not been resolved. Finally, RORα target genes, and mechanism of action in this metabolically demanding major mass lean tissue remained unclear.

The study by Mamontova et. al. (40) demonstrated the RORα sg/sg mice have lowered plasma HDL cholesterol. We demonstrate that the muscle cells with attenuated RORα expression and function display decreased ABCA1 mRNA expression. Furthermore, we demonstrated that muscle specific caveolin-3 mRNA expression is compromised in the cell line expressing RORΔDE. In addition, we demonstrated that the caveolin-3 gene is a direct RORα target in muscle cells. While caveolin-3 has not been directly linked to lipid regulation, studies of the non-muscle caveolin family member, caveolin-1, have suggested a role for caveolins in cholesterol transport to extracellular cholesterol acceptors such as HDL; increased caveolin-1 expression increases cholesterol efflux to extracellular acceptors. Furthermore, ApoA-I and cholesterol acceptor availability regulates caveolin-1 expression (41,42). Non-striated muscle caveolins have also been linked to other aspects of lipid regulation; caveolin-1 and –2 associate with lipid droplets in a fatty acid-regulated fashion in cultured cells and in vivo (43). Moreover, caveolin-1 null mice are resistant to diet-induced obesity (44) and show defective lipid droplet formation in a model system (Cohen). Recent studies showed that caveolin-1 expression increases, and caveolin-2 expression decreases, in white adipose tissue from rodents on a high fat diet (45). The studies presented here linking RORα to the regulation of caveolin-3 and ADRP mRNA expression, raises the intriguing possibility that like the non-muscle caveolins, caveolin-3 may play a role in lipid regulation. This is consistent with studies showing that a caveolin-3 mutant associates with lipid droplets...
and can perturb the cellular balance of neutral lipids and free cholesterol in cultured cells (43,46).

We further observed in the cell line expressing ectopic dominant negative RORα that expression of genes involved in lipid absorption, lipid catabolism, lipogenesis and energy expenditure was significantly repressed. This provides further evidence that RORα is a critical regulator of lipid homeostasis. Moreover, the repression of SREBP-1c expression and its target genes (for example FAS, SCD-1 and –2) in the muscle cell line expressing the truncated RORα similar in size and function to the form in the staggerer mouse, is consistent with the reduced plasma triglycerides observed in this animal model. In addition the staggerer mouse has lower apoCIII expression in the liver, which affects lipoprotein metabolism.

The neural degeneration, motor and coordination (i.e. spatial memory) abnormalities, in staggerer mice are consistent with the aberrant lipid metabolism observed. Defects in beta oxidation and lipid turnover are associated with neurologic disease (47).

We demonstrate that RORα has a significant role in the regulation of the mRNAs encoding the uncoupling proteins (UCP-2 and -3, mitochondrial proton carriers) that control metabolic efficiency, energy expenditure, and thermogenesis by uncoupling oxidation/respiration induced proton gradient from ATP synthesis. The repression of UCP mRNA expression correlates, and is in concordance with the decreased mRNA expression of genes involved in absorption and utilisation of lipids and fatty acids. For example, reduced lipoprotein lipase activity in skeletal muscle results in decreased UCP-3 mRNA expression, consistent with the hypothesis that it regulates the flux of lipid substrates across the mitochondria. Moreover, inhibition of fatty acid oxidation leads to reduced UCP-2 and 3 mRNA expression in slow twitch oxidative muscle fibres. Staggerer mice are lean despite
their hyperphagia (like cav1 mice). The lean phenotype may correlate with the decreased levels of SCD-1 and 2 mRNA observed, although increased brown adipose tissue activity may account for this phenotype.

In addition, we demonstrate that M-CPT-1 an established target for PPARα in cardiac muscle (26,48,49) and PPARβ/δ in skeletal muscle cells, is also directly regulated by RORα, and coactivates by p300, and PGC-1. Moreover, it suggests crosstalk may occur between PPAR and ROR α signalling in a tissue specific manner.

The fact that RORα is involved in all of these processes highlights the crucial role of RORα in lipid homeostasis and identifies RORα as a promising therapeutic target in the treatment of dyslipidemia. Skeletal muscle is rapidly emerging as a critical target tissue in the battle against obesity, type II diabetes, dyslipidemia, syndrome X and atherosclerosis. For example, NRs in skeletal muscle, such as LXR, PPARα, β/δ and γ have been shown to be involved in enhancing the insulin stimulated glucose disposal rate, decreasing triglycerides and increasing lipid catabolism, cholesterol efflux and plasma HDL-C levels (21-23). Hence, orphan NRs (for example ROR) that regulate lipid and carbohydrate metabolism, energy expenditure and thermogenesis in skeletal muscle have enormous pharmacological utility for the treatment of dyslipidemia, and syndrome X. In conclusion, we suggest that ROR in skeletal muscle cells programs a cascade of gene expression designed to regulate lipid homeostasis. We hypothesize that RORα is a critical regulator of lipid, and energy homeostasis in this major mass lean tissue. Moreover, we suggest that the atherogenic and dyslipidemic phenotype of the staggerer mouse is related to aberrant RORα function in skeletal muscle. Finally, we surmise that ROR agonists may have therapeutic utility in the treatment of hypercholesterolemia, atherosclerosis and obesity.
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Table 1: Key target genes in this study.

| Gene/Complex                                                                 | Description                                                                                                                                                                                                 |
|------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ABCA1 & ABCG8                                                                | ATP binding cassette: Transporters that transfer cholesterol to the HDL acceptors, i.e. reverse cholesterol efflux.                                                                                           |
| ACS4                                                                         | Acyl-CoA synthetase-4. Enhances the uptake of fatty acids by catalysing their activation to acyl-CoA esters for subsequent use in catabolic fatty acid oxidation pathways.                                            |
| ADRP / Adipophilin                                                           | Adipocyte differentiation-related protein. Involved in lipid storage.                                                                                                                                                                                                 |
| AdiopR1 & R2                                                                 | Adiponectin Receptors 1 & 2. Cell surface membrane receptors for adiponectin that regulate glucose uptake and fatty acid oxidation (Yamaguchi et al, 2003).                                      |
| ApoE                                                                         | Apolipoprotein-E: Facilitates cholesterol and lipid efflux.                                                                                                                                                                                                           |
| CAV3                                                                        | Caveolin 3. Muscle specific caveolin involves in membrane protein anchoring, caveolae, t-tubules formation and lipid trafficking.                                                                               |
| CPT-1                                                                       | Carnitine palmitoyl transferase-1. Transfers the long-chain fatty acyl group from coenzyme A to carnitine, the initial reaction of mitochondrial import of long-chain fatty acids and their subsequent oxidation. |
| FAS                                                                          | Fatty acid synthase. Involved in de novo fatty acid production.                                                                                                                                                                                                       |
| FAT/Fatty acid translocase& FABP3                                            | Fatty acid translocase, and fatty acid binding protein. Facilitate uptake of long chain fatty acids (LFCAs) & low dense lipoproteins (LDLs).                                                                   |
| GLUT-4 & -5                                                                 | Glucose transporters. GLUT4 facilitates glucose uptake in response to insulin stimulation. GLUT5 catalyzes uptake of fructose.                                                                                   |
| Glycogenin / GYG1                                                            | Initiates the synthesis of glycogen, the principal storage form of glucose in skeletal muscle.                                                                                                                                                                          |
| LPL                                                                          | Lipoprotein lipase. Hydrolysis of lipoprotein triglycerides into free fatty acids and responsible for the uptake of free fatty acids.                                                                             |
| PDK-2 & -4                                                                   | Pyruvate dehydrogenase kinases: Inhibiting the pyruvate dehydroxygenase complex, thereby controlling glucose oxidation and maintaining pyruvate for gluconeogenesis.                                             |
| SCD-1 & -2                                                                  | Stearoyl CoA desaturase-1, & -2. Enzymes associated with adiposity, i.e. storage and esterification of cholesterol, and responsible for the cis saturation of stearoyl and palmitoyl-CoA converting them to olate and palmitoleate, which are the monounsaturated fatty acids of triglycerides. |
| SREBP-1c                                                                     | Sterol regulatory element binding protein-1c, the hierarchical transcriptional activator of lipogenesis.                                                                                                      |
| UCP-1, -2 & -3                                                               | Uncoupling proteins. Mitochondrial proteins that uncouple metabolic fuel-oxidation from ATP-synthesis, regulating energy expenditure.                                                                         |
Figure Legends:

Figure 1. ROR mRNA expression in skeletal muscle cells.

A) Schematic illustration of the cell culture model. Proliferating myoblasts are cultured in DMEM supplemented with 10% serum supreme. Myoblasts were differentiated into post-mitotic multinucleated myotubes by 1-5 days of serum withdrawal (i.e. cultured in DMEM supplemented with 2% horse serum) B-J) Quantitative Real Time (Q-RT) PCR analysis of RORα and γ mRNA expression during skeletal myogenesis. Total RNA was extracted from C2C12 proliferating myoblasts (PMB), confluent myoblasts (CMB), myotube (MT) after 2 days of serum withdrawal (MT2) and myotubes after 5 days of serum withdrawal (MT5), and 7µg of each sample was reverse transcribed to cDNA. Subsequently, 0.6 percent of the cDNA was analysed by Q-RT PCR (see Materials and Methods). B) Q-RT PCR analysis of mRORα mRNA levels; C) Northern analysis of RORα mRNA levels as previously reported (19); Q-RT PCR analyses of D) mRORγ mRNA levels, E) mRNA levels (MyoG), F) β-actin mRNA levels, G) troponin I slow/type 1 (TNNI1) mRNA levels, H) troponin I fast/type II (TNNI2) mRNA levels, I) RVR mRNA levels and J) Rev-erbα mRNA levels. (Primer sequences and experimental conditions for Q-RT PCR are described in the materials and methods section).

Levels of 18S were measured in all samples, the results normalized and presented as number of target transcripts per 18S transcript. For example beta actin is equivalently expressed to 18S.

Figure 2. RORα-1-235/ RORΔDE attenuates ROR dependent gene expression.

A) Diagrammatic representation of RORalpha and the Gal4-ROR chimeric constructs. Each well of a six well plate of COS-1 cells (~60% confluence) were contransfected with 1.0µg of the Gal4-ROR chimera and 3.0 µg of the GAL reporter G5E1b-CAT by the liposome mediated transfection procedure as described in the materials and methods. Briefly, the DNA/liposome mix was added to the cells in 3 mls of DMEM supplemented with 5% charcoal stripped fetal calf serum. 16-24h later, the culture medium was changed, and the
cells were subsequently harvested for the assay of CAT enzyme activity 24-48h after the transfection period. Fold activation is expressed relative to CAT activity obtained after cotransfection of the Gal4 DNA-binding domain alone, arbitrarily set at 1. The mean fold activation values and standard deviations (bars) were derived from a minimum of 2 independent triplicate experiments.

B) Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with either 0.33ug of the synthetic reporter, RORE x5-TKLuc [the RORE sequence are described in the materials and methods]. Additionally, either 0.32 µg of pSG5, or 0.16ug each of pSG5-RORa and pSG5, or 0.16ug each of pSG5-RORa1 and pSG5-RORΔDE or 0.16ug each of pSG5-RORa and pSG5-RVR (i.e. a total of ~ 0.65 µg DNA per well) using the liposome mediated transfection procedure as described in A). Fold activation is expressed relative to LUC activity obtained after cotransfection of the reporter, and pSG5 vector only, arbitrarily set at 1. The mean fold activation values and standard deviations (bars) were derived from a minimum of 2 independent experiments comprised of six replicates.

C) Identical to B), except the native reporter, mPCP-2 x4-TKLuc has been utilized

D) and E) COS-1 cells were transiently transfected as above with 0.32ug of the synthetic reporter, ROREx5-TKLuc. The control transfection arbitrarily set to 1, also contained 0.32 ug of the pSG5 vector alone. All the other transfections contained 0.16ug pSG5-RORa1 per well of a 24 well plate. Additionally, the three denoted were cotransfected with increasing amounts (0.04, 0.08 and 0.16 ug) of pSG5-RORΔDE (D) and pSG5-RVR (E), respectively. The vector, pSG5 was used to standardize the quantity of DNA to a total of ~ 0.65 µg DNA per well. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the reporter with the pSG5 vector, alone, arbitrarily set at 1. The mean Luciferase fold activation values and standard deviations (bars) were derived from a minimum of 2-3 independent experiments comprised of six replicates.
Figure 3. Quantitative Real Time PCR analyses of the RORΔDE stably transfected C2C12 cell line

Quantitative Real Time PCR analysis of endogenous and exogenous/ectopic ROR mRNA expression in two independent populations of native/wild type (wt) differentiated mouse C2C12 cells, and two independent polyclonal pools of the C2C12 cells stably transfected with the dominant negative human RORα1ΔDE expression vector, after five days of serum withdrawal. These cells were denoted as wt C2’s, and C2:RORΔDE, respectively.

A) Q-RT PCR analysis of the dominant negative human RORα (hROR) mRNA expression in the two independent populations of wild type mouse C2C12 cells, and the two independently transfected polyclonal pools of the C2:RORΔDE cells relative to the GAPDH control, and B) Q-RT PCR analysis of hRORα mRNA expression relative to the 18S rRNA control. C) Northern analysis of the dominant negative human RORα mRNA as previously reported (19). Total RNA from proliferating myoblasts (PMB), and myotubes after 2 days of serum withdrawal (MT2) was isolated from wt and stably transfected cells. Subsequently, RNA was northern blotted and hybridized with a 32-P labelled human ROR probe, and an oligo that recognises the 18S rRNA. D) Q-RT PCR analysis of total mouse + human RORα mRNA levels (m+hROR) using primers that recognise the mouse and human RORs. E) Q-RT PCR analysis of mouse RORα (mROR) that measure the levels of the endogenous ROR mRNA levels. F) Northern analysis of the endogenous mouse RORα as previously reported (. G) Q-RT PCR analysis of mouse RORγ (mROR) that measure the levels of the endogenous ROR mRNA levels. Specific primer sequences and PCR conditions are described in the Materials and Methods section).

Figure 4 Expression Profiling of mRNAs involved in metabolism by Q-RT PCR analyses of the wild type native C2C12 cells, and the C2:RORΔDE stably transfected cells.

Quantitative Real Time PCR analysis of endogenous mRNA expression encoding enzymes/proteins involved in lipid homeostasis, energy expenditure etc (described in table 1) in two independent populations of native/wild type (wt) differentiated mouse C2C12 cells, and two independent polyclonal pools of the C2C12 cells stably transfected with the dominant negative human RORα1ΔDE expression vector, after five days of serum withdrawal. These
cells were denoted as wt C2’s, and C2:RORΔDE, respectively. Levels of 18S were measured in all samples, the results normalized and presented as the number of target transcripts per 18S transcript. For example beta actin is equivalently expressed to 18S.

A-C) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in lipid absorption that are completely described in Table 1. A) Fatty Acid Translocase (CD36/FAT), B) Fatty Acid Binding Protein 3 (FABP3) and C) FABP4. D and E) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in glucose uptake that are completely described in Table 1. D) Glucose transporter 4 (GLUT4), and E) GLUT5. F-K) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in lipid catabolism that are completely described in Table 1. F) Lipoprotein Lipase (LPL), G) Carnitine Palmitoyl Transferase-1(CPT-1), H) Acyl Co-A Synthetase-4 (ACS-4), I) and J) Adiponectin Receptors 1 and 2 (adipoR1 and R2), K) PPAR Gamma Coactivator-1 (PGC-1). L and M) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in energy expenditure that are completely described in Table 1. L) Uncoupling Protein-2 (UCP-2), M) UCP-3. N-R) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in lipid efflux and storage that are completely described in Table 1. N) ATP Binding Cassette transporter A1 (ABCA1), O) ABCA8/G1, P) Apolipoprotein E (ApoE), Q) Caveolin 3 (CAV3) and R) Adipocyte differentiation related protein/adipophilin (ADRP). S-V) Q-RT PCR analysis of mRNA expression encoding proteins/enzymes involved in lipogenesis that are completely described in Table 1. S) Sterol Regulatory Element Binding Protein-1c (SREBP-1c), T) Stearoyl CoA Desaturase-1 (SCD-1), U) SCD-2, V) Fatty Acid Synthase (FAS). W-Z, Aii and Bii) Q-RT PCR analysis of mRNA expression encoding proteins associated with differentiation of the C2:RORΔDE cell line, i.e. Myogenin (MyoG). b-actin, troponin I type I/slow (TNNI1) and troponin I type II/fast (TNNI2). W) MyoG, X) b-actin, Y), MyoG, Z) b-actin, Aii) TNNI1, Bii TNNI2..

Figure 5 Quantitative Real time PCR analyses of orphan nuclear receptor expression in the hRORΔDE stably transfected C2C12 cell line.

Quantitative Real Time PCR analysis of endogenous mRNAs encoding the closely related Rev-erb and RVR orphan nuclear receptors in two independent populations of native/wild type (wt) differentiated mouse C2C12 cells, and two independent polyclonal pools of the
C2C12 cells stably transfected with the dominant negative human RORAΔE expression vector, after five days of serum withdrawal. These cells were denoted as wt C2’s, and C2:RORAΔE, respectively.

Q-RT PCR analyses of A) RVR mRNA expression and B) Rev-erbα mRNA expression in wt C2’s and C2:RORAΔE cells. Specific primer sequences and conditions for RVR and Rev-erb Q-RT PCR are described in the materials and methods. Levels of 18S were measured in all samples, and the results presented as number of target transcripts per 18S transcript. For example beta actin is equivalently expressed to 18S.

**Figure 6** PGC1 acts as a transcriptional coactivator for ROR-mediated activation of the heterologous synthetic and native RORE reporter genes.

Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33ug of the synthetic reporter (i.e the RORE x 5-tk-Luc containing 5 copies of the TATATCAAGGTCAT sequence), 0.33ug of either pSG5, or pSG5-RORα1, and 0.1ug pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~0.7 µg DNA per well) using the liposome mediated transfection procedure as described previously. Fold activation is expressed relative to LUC activity obtained after cotransfection of the reporter, and vector only, arbitrarily set at 1. The mean fold activation values and standard deviations (bars) were derived from a minimum of 2 independent experiments comprised of six replicates.

**B)** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33ug of the native reporter (i.e the mouse PCP-2x4-tk-LUC contain 4 copies of the native sequence GTTATAGTAATGGTCAGGGGACT), 0.33ug of either pSG5, or pSG5-RORα1, and 0.1ug pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~0.7 µg DNA per well) using the liposome mediated transfection procedure as described.

**C)** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33ug of the tk-LUC vector, 0.33ug of either pSG5, or pSG5-RORα1, and 0.1ug pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~0.7 µg DNA
per well) using the liposome mediated transfection procedure as described previously, and above.

**Figure 7 The M-CPT1-promoter is directly activated by ROR, and coactivated by p300 and PGC-1.**

**A:** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with either 0.33 μg pGL2-basic, pGL2-MCPT1_(−1025/−12) or pGL2-GLUT4, and 0.33 μg of either pSG5, or pSG5-RORα1 (i.e. a total of ~0.66 μg DNA per well) using the liposome mediated transfection procedure as described previously, and above (Figure 6A).

**B** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33 μg pGL2-MCPT1_(−1025/−12), 0.33 μg of either pSG5, or pSG5-RORα1, and 0.1 μg pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~0.7 μg DNA per well) using the liposome mediated transfection procedure as described.

**C** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33 μg pGL2-MCPT1_(−1025/−12), 0.33 μg of either VP16 vector alone, or the VP16-RORα1 chimera, and 0.1 μg pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~0.66 μg DNA per well) using the liposome mediated transfection procedure as described.
**Figure 8:** The Caveolin 3 promoter is directly activated by ROR\(\alpha\), and coactivated by p300 and SRC-2.

**A)** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with either 0.33\(\mu\)g pGL3-basic and a series of mouse pGL3-mCav3 promoter deletions –2595/+35 (2595), -988/+35 (988), -654/+35 (654), -477/+35 (477), -279/+35 (279), and 0.33\(\mu\)g of either pSG5, or pSG5-ROR\(\alpha\)1 (i.e. a total of ~ 0.66 \(\mu\)g DNA per well) using the liposome mediated transfection procedure as described above.

**B)** Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.33\(\mu\)g pGL3-mCav3-LUC (-2595/+35), 0.33\(\mu\)g of either pSG5, or pSG5-ROR\(\alpha\)1, and 0.1\(\mu\)g pcDNA expression vectors encoding p300, SRC-2/GRIP-1 and PGC-1.(i.e. a total of ~ 0.7 \(\mu\)g DNA per well) using the liposome mediated transfection procedure as described above

**C)** Sequence of putative RORE motifs (in bold) identified in the mouse Caveolin 3 promoter. TT below the line denotes mutagenesis of the bases above from GG to TT by Quickchange site directed mutagenesis referred to in Figure 8F.

**D)** Electrophorectic Mobility Shift Analysis (EMSA) analysis examining the differential interaction of the six putative RORE motifs in the Caveolin3 promoter (denoted as Cav3 motifs (m) 1-6) with GST-ROR\(\alpha\)1. 300cps of PAGE purified, \(^{32}\)P- end radiolabeled Cav3 m1-6 were incubated with 0.4\(\mu\)g GST-ROR\(\alpha\)1 protein before PAGE separation. The mPCP-2 radiolabeled probe was used as a positive control (see Methods).

**E)** EMSA analysis examining the ability of the six putative m1-m6 RORE’s (at 10- and 30-fold excess) to efficiently compete the mPCP-2/ROR complex. \(^{32}\)P- radiolabeled mouse PCP-2 RORE probe was co-incubated with 0.4\(\mu\)g GST-ROR\(\alpha\)1 protein, and 100ng and 300ng unlabeled putative Cav 3 m1-m6 RORE’s before PAGE separation. Cold mPCP-2 and synthetic RORE’s were used as the control competitors(see Methods).
F) Each well of a 24 well plate of COS-1 cells (~60% confluence) was co-transfected with 0.4ug pGL3-mCav3-LUC (-2595/+35) or pGL3-mCav3-LUC (-2595/+35) carrying the GG to TT mutations in motifs 2 and 6 (see figure 8C) in the presence 0.1ug pcDNA or pcDNA-p300 and 0.16ug pSG5 or pSG5-RORα1 as described in Methods section.
**A)** Skeletal muscle cell culture model

- Proliferating myoblasts (PMB)
- Post-mitotic differentiated myotubes (MT)

**B)**

- Harvest RNA after 2 and 5 days of differentiation
- Real Time PCR

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**C)**

- Image of a gel with bands labeled as RORα and 18S RNA

**D)**

- Bar chart of normalized mRORγ mRNA/18S rRNA across PMB, CMB, MT2, and MT5

**E)**

- Bar chart of normalized Myogenin mRNA/18S rRNA across PMB, CMB, MT2, and MT5

**F)**

- Bar chart of normalized β-Actin mRNA/18S rRNA across PMB, CMB, MT2, and MT5

**G)**

- Graph showing normalized mRNA/18S rRNA for Troponin I slow across PMB, CMB, MT2, and MT5

**H)**

- Graph showing normalized mRNA/18S rRNA for Troponin I fast across PMB, CMB, MT2, and MT5

**I)**

- Graph showing normalized mRNA/18S rRNA for RVR/Rev-erb β across PMB, CMB, MT2, and MT5

**J)**

- Graph showing normalized mRNA/18S rRNA for Rev-erb α across PMB, CMB, MT2, and MT5
A) hRORα
B) hRORα
C) hRORα
D) m+hRORα
E) mRORα
F) mRORα
G) mRORα
A) pGL3-mCav3-LUC

| pSG5 | RORα1 |
|------|-------|
| 7.5  | 5.0   |
| 5.0  | 2.5   |
| 2.5  | 1.0   |
| 1.0  | 0.0   |

2595 988 654 477 279 pGL3b

B) RLU's

| pcDNA | p300 | SRC-2 | PGC-1 |
|-------|------|-------|-------|
| 5.0x10^4 | 4.0x10^4 | 3.0x10^4 | 2.0x10^4 |
| 4.0x10^4 | 3.0x10^4 | 2.0x10^4 | 1.0x10^4 |

pSG5 pSG5-RORα1

C) Competitor:

1. Cav3m1
-2498 to -2474
CTGAGAACTACGTCATGGCCTTTTG
GACTTGGATGCAAGTACCGGAAC

2. Cav3m2
-2378 to -2357
CTCCTGACCTACTACCCGG
GACGACAGTGAGTCAGTGAGGC

3. Cav3m3
-1739 to -1713
CTGCCAAGGCACAGGAGACACGC
GACGAGGTCCGTTCTCCTCGTGCTG

4. Cav3m4
-1720 to -1690
AGCAGACAGGATCTTTGTCCTTTTGTT
TCGTCGCTTCCAGAAACACAGGAAACACAG

5. Cav3m5
-1587 to -1556
CATGGAACAGGTAAAAGGGAGAGTTAACTGA
GTACCTGTTCCATTTCCCTCCTCGGAATTTGAC

6. Cav3m6
-1005 to -984
CCAGTGACCACCTCCTCTCT
GGTCATCQGCTGAAAGGAGGAGA

D) GST-RORα

E) Probe: PCP-2

| m1 | m2 | m3 | m4 | m5 | m6 | RORE | C |
|----|----|----|----|----|----|------|---|

F) Fold Activation

| pcDNA | p300 | p300 | p300 | p300 |
|-------|------|------|------|------|
| 1.0  | 1.0  | 1.0  | 1.0  | 1.0  |
| 2.0  | 2.0  | 2.0  | 2.0  | 2.0  |
| 3.0  | 3.0  | 3.0  | 3.0  | 3.0  |
| 4.0  | 4.0  | 4.0  | 4.0  | 4.0  |
| 5.0  | 5.0  | 5.0  | 5.0  | 5.0  |
ROR α regulates the expression of genes involved in lipid homeostasis in skeletal muscle cells: Caveolin-3 and CPT-1 are direct targets of ROR
Patrick Lau, Susan J. Nixon, Robert G. Parton and George E. O. Muscat

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