The Orphan Nuclear Receptor, ROR\(\alpha\), Regulates Gene Expression That Controls Lipid Metabolism

**STAGGERER (SG/SG) MICE ARE RESISTANT TO DIET-INDUCED OBESITY**

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Patrick Lau, Rebecca L. Fitzsimmons, Suryaprakash Raichur, Shu-Ching M. Wang, Adriane Lechtken, and George E. O. Muscat

From the Institute for Molecular Bioscience, University of Queensland, Queensland 4072, Australia

Homozygous staggerer mice (sg/sg) display decreased and dysfunctional retinoic acid receptor-related orphan receptor \(\alpha\) (ROR\(\alpha\)) expression. We observed decreases in serum (and liver) triglycerides and total and high density lipoprotein serum cholesterol in sg/sg mice. Moreover, the sg/sg mice were characterized by reduced adiposity (associated with decreased fat pad mass and adipocyte size). Candidate-based expression profiling demonstrated that the dyslipidemia in sg/sg mice is associated with decreased hepatic expression of SREBP-1c, and the reverse cholesterol transporters, ABCA1 and ABCG1. This is consistent with the reduced serum lipids. The molecular mechanism did not involve aberrant expression of LXR and/or ChREBP. However, ChIP and transfection analyses revealed that ROR\(\alpha\) is recruited to and regulates the activity of the SREBP-1c promoter. Furthermore, the lean phenotype in sg/sg mice is also characterized by significantly increased expression of PGC-1\(\alpha\), PGC-1\(\beta\), and lipin1 mRNA in liver and white and brown adipose tissue from sg/sg mice. In addition, we observed a significant 4-fold increase in \(\beta_2\)-adrenergic receptor mRNA in brown adipose tissue. Finally, dysfunctional ROR\(\alpha\) expression protects against diet-induced obesity. Following a 10-week high fat diet, wild-type but not sg/sg mice exhibited a \(\sim20\%\) weight gain, increased hepatic triglycerides, and notable white and brown adipose tissue accumulation. In summary, these changes in gene expression (that modulate lipid homeostasis) in metabolic tissues are involved in decreased adiposity and resistance to diet-induced obesity in the sg/sg mice, despite hyperphagia. In conclusion, we suggest this orphan nuclear receptor is a key modulator of fat accumulation and that selective ROR modulators may have utility in the treatment of obesity.

The spontaneously arising mouse mutant, staggerer (sg), was initially described and analyzed several decades ago (1).
**RORα Regulates Adiposity**

RORα is abundantly expressed in the liver and the other major mass peripheral metabolic tissues. Moreover, sg/sg mice consume an increased amount of food (per gram bodyweight), but, despite this feeding pattern, they maintain a lower weight (15, 16). Surprisingly, no studies have investigated this aspect of the staggerer phenotype. Hence, we were particularly interested in elucidating the functional role of RORα in the control of genes that regulate lipogenesis and fatty acid oxidation in liver, skeletal muscle, and white adipose tissues in homozygous sg/sg mice. We hypothesized, based on our in vitro studies, that SREBP-1c may be a critical component in the metabolic phenotype of sg/sg mice.

We observed that sg/sg mice displayed an early onset decrease in weight that was maintained after maturity and associated with decreased adiposity. The mice (expressing dysfunctional RORα) displayed reduced serum and hepatic lipids, consistent with decreased ATP-binding cassette, subfamily A, member 1 (ABCA1), ATP-binding cassette, subfamily G, member 1 (ABCG1), SREBP-1c, and fatty acid synthase (FAS) mRNA expression in the liver. ChIP and transfection analyses showed that RORα-mediated trans-activation of the SREBP-1c promoter. Candidate-based expression profiling in sg/sg mice demonstrated increased expression of peroxisome proliferator-activated receptor-γ, coactivator 1 (PGC-1α/β) and lipin 1 gene expression in liver and white and brown adipose tissue. These genes increase fatty acid utilization and oxidative metabolism. Interestingly, the sg/sg mice did not accumulate fat and were resistant to diet-induced obesity, despite hyperphagia. In summary, our study suggests RORα is an important factor in the regulation of genes associated with lipid homeostasis and adiposity.

**MATERIALS AND METHODS**

**Animals and Tissue Collection**—Heterozygous +/+sg mice, B6.C3(Cg)-Rora<sup>+/J</sup>, were obtained from Jackson laboratory (Bar Harbor, ME), and the colony was maintained by backcrossing to C57BL/6J. Identification was carried out by PCR genotyping as described by Jackson laboratory. The mice were housed in Queensland Bioscience Precinct vivarium (University of Queensland, St. Lucia, Queensland, Australia) with a 12-h light-dark cycle and fed a standard diet containing 4.6% total fat or a high fat diet (SF01-028) containing 22.6% fat (both from Specialty Feeds, Glen Forrest, Western Australia). Heterozygous sg/sg mice and their wild-type (wt) littermates were obtained by crossing heterozygous male and female breeders. The mice were weaned at 4 weeks of age but were fed with moist mash standard diet ad libitum as described by Guastavino (17) in the final week prior to weaning and thereafter postweaning. High fat fed animals were similarly provided with moist, mashed pellets and diluted, food grade strawberry flavored to improve palatability. Access to water was facilitated by adding dishes of water and hanging gel packs (Able Scientific, Welshpool, Western Australia) in each cage. Experimental animals were weighed weekly. Mice were fasted for 6 h by transferring to a new food-free holding cage with unrestricted access to water. Blood collection was performed by terminal cardiac puncture, under isoflurane anesthesia. Otherwise animals were euthanized by cervical dislocation at 14 weeks of age. Tissues collected for RNA extraction were immediately frozen in liquid nitrogen and then stored at −80 °C. For histological examination, tissues were fixed in 10% buffered formalin (Sigma-Aldrich), paraffin-embedded, and sections were stained with hematoxylin and eosin. All aspects of animal experimentation were approved by The University of Queensland Animal Ethics Committee.

**RNA Extraction and cDNA Synthesis**—Total RNA extraction and cDNA synthesis were performed as previously described, with minor modifications. Following homogenization with a handheld ultra-turrax homogenizer (IKA, Staufen, Germany), total RNA from liver and quadriceps muscles was extracted using TRI reagent (Sigma-Aldrich) and an RNeasy mini kit (Qiagen) according to the manufacturers’ protocol. For epididymal adipose tissue and brown adipose tissue, RNA was extracted with Qiazol and an RNeasy mini kit (Qiagen). Reverse transcription was performed as previously described, using 1 μg of total RNA in each cDNA synthesis reaction (14).

**Primers and Quantitative PCR**—Relative expression of genes was determined using the ABI 7500 Real-Time PCR System (ABI, Singapore) as previously described (14). Primers for 18S, total mRORα, ABCA1, ABCA8/G1, FAS, LPL, NOR1, NUR77, SREBP-1c, UCP2, MCAD, and CPT1b have been reported (14, 18, 19). ADRB1 (Mm00431701_s1), ADRB2 (Mm02524224_s1), ADRB3 (Mm00426691_m1), ATGL (Mm00503040_m1), HSL (Mm00495359_m1), LIPC (Mm00433975_m1), Lipin1 (Mm00550511_m1), GCK (Mm00439129_m1), Nur1 (Mm00430600_m1), CPT1a (Mm00504348_m1), PGC-1α (Mm00447183_m1), and PGC-1β (Mm00504720_m1) TaqMan Gene Expression Assays were obtained from Applied Biosystems (Foster City, CA). *Mus musculus* primer sequences (forward and reverse, respectively) used for SYBR assays are as follows: mRORα1, GGCAGGCGACGGACGGGAGCTATGC and CACGTAAATGACACCATATAATTGGT; mRORα2, GGGCCTAAAAGAGATGTATTTGTG and CACCAGCATTGCATGGAATAATT; ChREBP, GGAAGATCTCGGCTGAG and GCTTCTGCTCGTGTGTTCA; Lxrα, GAAATGCCTCCAGAGGAGTCTGAC and GATCTGTTCCTTCTGACAGCACA; Lxrβ, TGCAAACCATGTTTCTTCC and TCTCGGAAGCTGAGGTCTGCTG; UCP1, ACAGAAGAGTGGCAGC and AGCTGATTTGTCCTCTGAATG; UCP3, CTCCCCTTGGGACATTGACG and CAAAGAAGGGCCACCAATCC; and leptin, TCTGAACGATGCTTATCCGAA and GGTATCGACTGCGTGTG. Primers were obtained from Generewks (Thebarton, South Australia).

**Chromatin Immunoprecipitation**—Mouse myogenic C2C12 cells were differentiated into post-mitotic multinucleated myotubes by incubation for 5 days in Dulbecco’s modified Eagle’s medium (supplemented with 2% horse serum). Following homogenization with a handheld ultra-turrax homogenizer (IKA, Staufen, Germany), total RNA from liver and quadriceps muscles was extracted using TRI reagent (Sigma-Aldrich) and an RNeasy mini kit (Qiagen) according to the manufacturers’ protocol. For epididymal adipose tissue and brown adipose tissue, RNA was extracted with Qiazol and an RNeasy mini kit (Qiagen). Reverse transcription was performed as previously described, using 1 μg of total RNA in each cDNA synthesis reaction (14).

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**Chromatin Immunoprecipitation**—Mouse myogenic C2C12 cells were differentiated into post-mitotic multinucleated myotubes by incubation for 5 days in Dulbecco’s modified Eagle’s medium (supplemented with 2% horse serum). Subsequently, cells were washed twice in ice-cold phosphate-buffered saline and cross-linked in 1% formaldehyde solution. Chromatin immunoprecipitation was performed as described by Li et al. (20) using anti-RORα antibody (sc-6062, Santa Cruz Biotechnology, Santa Cruz, CA). The following SREBP1c quantitative PCR ChIP primers were used: NR1, CTTCAGATGTCAGAGCAGAGT and GTTCCTGTCCAGGCTGACG; NR2, CCCGCCCTCTTGGAAACAGT and GCAGCGAAGTTGTGCTACAGT; and glyceraldehyde-3-phosphate dehydrogenase, GCTCACTGGGAGTCGTTTCGACG.
Transient Transfections—Each well of a 24-well plate of COS-1 or C2C12 cells was transfected with a total of 0.6–0.8 μg of DNA per well using the liposome-mediated transfection procedure. Cells were cotransfected with pGL3-SREBP1c luciferase gene reporter (21) and either pSG5-RORα1 (22), pSG5-RORα4 (2), or empty vector using a N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate and Metaflectene (Biontex Laboratories GmbH, Munich, Germany) liposome mixture as described previously (14). The -fold activation is expressed relative to LUC activity obtained after cotransfection of the empty reporter and pSG5 vector only, arbitrarily set at 1. The mean -fold activation values and standard error were derived from three independent experiments of triplicate wells.

Blood and Tissue Analysis—High density lipoprotein cholesterol from whole blood was measured using the Cholestech LDX System (Hayward, CA). Measurements of total cholesterol, triglyceride, and non-esterified fatty acid in heparinized plasma samples were analyzed by Clinical Pathology Laboratory, School of Veterinary Science, The University of Queensland (St. Lucia, Queensland, Australia). To obtain hepatic triglyceride measurements, liver tissue was saponified by digestion with ethanolic KOH, neutralized, and then assayed using Free Glycerol Reagent (Sigma-Aldrich).

Statistical Analysis—Data were analyzed (and significance assigned) using the ratio t test in the GraphPad Prism 4 software, unless otherwise indicated.

RESULTS

RORA1 and -A mRNA Expression Is Substantially Attenuated in the Metabolic Tissues from sg/sg Mice—As discussed, a number of studies have implicated RORα in the regulation of lipid homeostasis. We were particularly interested in exploring this further and examining the effect of functional RORα deficiency on the regulation of critical genes involved in anabolic and catabolic lipid metabolism in the major metabolic tissues in sg/sg mice.

Initially, we investigated ROAR1 and -α4 mRNA expression in the skeletal muscle, liver, and white adipose tissue of homozygous sg/sg mice. We demonstrated expression of the mRNAs encoding ROAR1 and -α4 was significantly attenuated in the muscle, liver, and adipose tissues of homozygous sg/sg mice (Fig. 1, A and B). This is concordant with previously reported analysis of ROARα expression in the cerebellum, liver, and intestine of sg/sg mice (5, 12, 23).

Staggerer (sg/sg) Mice Display an Early Onset (~20%) Decrease in Weight That Is Maintained after Maturity and Revealed Reduced Adiposity—Interestingly, as discussed in a very recent report (24), no detailed data (over many weeks) on the specific changes in body weight in sg/sg mice have been reported (relative to wild-type littermates), despite hyperphagia on a regular chow diet (16). Crucially, our mice were fed a “pasty mix” of crushed food moistened with water and placed on the floor of the cage. Water is supplied in a small dish, and accessible gel packs are provided in each cage. This circumvents any issues associated with access to food due to stretching difficulties in sg/sg mice that effects mortality (17). Subsequently, we ascertained the growth pattern of male sg/sg mice from age 4 weeks to maturity at 14 weeks on a normal chow diet.

At weaning, the sg/sg mice are ~50% smaller than their wt counterparts. As they mature the size difference lessens, and by adulthood male sg/sg mice maintain a body mass that is ~15–20% decreased relative to wt mice (Figs. 2, A and B). The sg/sg mice displayed reduced adiposity (Fig. 2C). Interestingly, the size of the epidymal white adipose depots (corrected for total body weight) were markedly and significantly decreased in sg/sg mice (Fig. 2D). This correlated with a substantial reduction in adipocyte cell size (Fig. 2E). No significant differences were observed in the heart, quadriceps, and gastrocnemius skeletal muscle tissues, however, a small, but significant decrease in standard weight was observed (Fig. 2D).

Decreased Plasma Cholesterol Is Associated with Decreased Expression of mRNAs Encoding the Reverse Cholesterol Transporters ABCA1 and ABCA8/G1 in the Liver—Mamontova et al. (11) previously reported decreased serum cholesterol in sg/sg mice that is associated with decreased apolA1 expression in the intestine (not the liver). We similarly observed plasma (total and HDL) cholesterol were decreased in the serum of sg/sg mice on a normal chow diet (Fig. 3, A and B). We utilized candidate-based expression profiling of the liver, and examined several additional genes (and pathways) that could potentially account for reduced plasma cholesterol. We identified significantly reduced expression of the mRNAs encoding the reverse cholesterol transporters, ABCA1, and ABCA8/G1 in the liver of sg/sg mice (Fig. 3, C and D). Many studies have reported the link between impaired hepatic reverse cholesterol transport and HDL cholesterol.

Decreased Serum and Liver Triglycerides in sg/sg Mice Are Mediated by Decreased SREBP-1c and FAS mRNA Expression in the Liver—Raspe et al. (13) demonstrated reduced plasma triglycerides in female sg/sg mice that was associated with reduced apoCIII expression in the liver. Analysis of male sg/sg mice revealed that triglycerides (and non-esterified fatty acids) were decreased (Fig. 4, A and B). Additionally, we examined the total triglyceride content and concentration of the liver. We report that total triglyceride content and the concentration (milligrams/g liver wet weight) are significantly reduced in the hepatic tissue from sg/sg mice, relative to wild-type littermates (Fig. 4C). This is consistent with decreased plasma triglycerides.

Subsequently, we utilized candidate-based profiling and examined several genes involved in lipogenesis to identify the
underlying changes in gene expression responsible for the dyslipidemia. SREBP-1c is an important hierarchical regulator of lipogenesis (25, 26). Changes in SREBP-1c (and target gene expression) in liver, adipose, and muscle are associated with changes in plasma triglycerides (25, 27). Interestingly, we observed significantly reduced expression of the mRNA encoding SREBP-1c (Fig. 4D) and the lipogenic enzyme, FAS (Fig. 4E) in the liver, and/or skeletal muscle of sg/sg mice, relative to the littermate wt controls. This is concordant with the observed (and reported) reduction in plasma triglycerides from sg/sg mice (13). In this context, as discussed above, we also observed gastrocnemius muscle, and liver of wt and sg/sg mice (each n = 8). Inset: photograph of epididymal fat pad of wt and sg/sg mice. Tissue weights are normalized against body weights. Results are expressed as the mean ± S.E. *, p < 0.05 and ***, p < 0.001. E, photomicrographs of sg/sg mice and wt littermate epididymal fat pad stained with hematoxylin and eosin.
RORα Regulates Adiposity

Reduced expression of the mRNA encoding ABCG1 in the liver of sg/sg mice (Fig. 3D), that is also consistent with reduced serum triglycerides and adiposity in the sg/sg mice. A very recent report (28), on the ablation of ABCG1 in mice, identified an unexpected role in adiposity and triglyceride storage.

Furthermore, in the context of decreased SREBP-1c expression and downstream target genes, we examined the expression of the mRNA encoding glucokinase in fasted liver and observed significantly reduced expression of Gck mRNA expression in sg/sg mice (Fig. 4F). In summary, decreased SREBP-1c and FAS mRNA expression observed in sg/sg mice account for the decreased serum (and liver) triglycerides and reduced adiposity.

RORα Regulates the SREBP-1c Promoter—Liver X receptors (LXRs) and carbohydrate response element-binding protein (ChREBP) (29, 30) are critical transcriptional regulators of SREBP-1c, lipogenic gene expression, and fatty acid biosynthesis. Hence, we investigated the expression of LXR (LXRα and LXRβ) and ChREBP in sg/sg mice. We did not detect major changes in the expression of these transcription factors, in the hepatic tissue of sg/sg, relative to wild-type mice (Fig. 4, G–I).

The change in LXRβ attained significance, however, the decrease in expression was very small. This suggested that the lower triglyceride levels in the liver and plasma were primarily the result of decreased SREBP-1c expression.

SREBP-1c expression is also regulated by insulin, repressed upon fasting, and tightly linked to the nutritional state (31–33). To date, C. Ron Kahn and colleagues (34) have recently stated “how these two factors (LXR and insulin) interact in the control of SREBP-1c remains a subject of debate” (35–37). In this context, we have observed in the fed versus (6 h) fasted transition that SREBP-1c mRNA expression is very similarly decreased in wt and sg/sg mice (data not shown).

This raised the question as to whether dysfunctional RORα expression is responsible for aberrant SREBP-1c expression in sg/sg mice. ChIP analysis (quantitated by real-time PCR amplification) in muscle cells demonstrated that RORα was efficiently and preferentially recruited to two upstream regions, NR1, between nucleotide positions −1342/−1158 and NR2 between nucleotide positions −481/−458 in the SREBP-1c promoter relative to the GAPDH promoter (see Fig. 5, A–C).

This suggested that RORα may regulate the expression of the SREBP-1c promoter. Transfection analysis demonstrated that RORα1 and -α4 trans-activated the SREBP-1c (−1200/+103) promoter in COS-1 (Fig. 5D) and C2C12 skeletal muscle cells (Fig. 5E).

The SREBP-1c (−1200/+103) promoter contained the NR2 region, the primary RORα recruitment site. However, our ChIP assay identified secondary recruitment of RORα to a region between nucleotides −1342 and −1158 (which contains three well conserved RGGTCA NR half-sites) (Fig. 5B) and was not

![FIGURE 4. Plasma lipids and gene expression in liver of male sg/sg and wt control mice.](image)

Analysis of plasma triglyceride (each n = 4) (A) and non-esterified fatty acid (each n = 4) (B) of male wt and sg/sg mice. C, measurement of total triglyceride content and triglyceride concentration in liver of male wt and sg/sg mice (n = 7–8). Quantitative PCR of (D) SREBP1c mRNA levels in liver and quadriceps muscle of male wt and sg/sg mice (n = 7–8). Quantitative PCR of FAS (E), GCK (F), LXRα (G), LXRβ (H), and ChREBP (I) mRNA levels in liver of male wt and sg/sg mice (n = 7–8). Relative mRNA expression is normalized against 185 rRNA. Results are expressed as the mean ± S.E. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.


RORα Regulates Adiposity

Subsequently, we examined PGC-1β expression in liver, white and brown adipose, and skeletal muscle tissue. Speigelman and colleagues (39, 40) have demonstrated PGC-1β induces the formation of oxidative (mitochondrial rich) muscle fibers and brown adipose tissue. We observed significantly elevated levels of PGC-1β mRNA in brown adipose tissue (Fig. 6H) (but not in liver, white adipose tissue, and skeletal muscle) of sg/sg mice relative to the littermate control wt mice (Fig. 6, B, E, and K).

Moreover, we examined lipin1 expression in liver, white and brown adipose, and skeletal muscle tissue. Finck et al. (41) have demonstrated that PGC-1α induces lipin1 expression in hepatic tissue and demonstrated that lipin1 selectively activates a subset of fatty acid oxidation pathways, while repressing lipogenesis and plasma lipids. We observed significantly elevated levels of lipin1 mRNA in liver and white and brown adipose tissue (but not in skeletal muscle) of sg/sg mice relative to the littermate control wt mice (Fig. 6, C, F, I, and L). The changes in lipin1 are in concordance with significantly increased PGC-1α expression, reduced lipogenic gene expression, and decreased (serum and liver) triglycerides.

In summary, we observed significantly increased expression of PGC-1α in liver, white and brown adipose and skeletal muscle tissue. Speigelman and colleagues (39, 40) have demonstrated PGC-1β induces the formation of oxidative (mitochondrial rich) muscle fibers and brown adipose tissue. We observed significantly elevated levels of PGC-1β mRNA in brown adipose tissue (Fig. 6H) (but not in liver, white adipose tissue, and skeletal muscle) of sg/sg mice relative to the littermate control wt mice (Fig. 6, B, E, and K).

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In addition, we examined the expression of genes directly involved in the fatty acid oxidation pathway (e.g. CPT-1a/b and MCAD) in liver, white adipose tissue, and skeletal muscle (Table 1). We observed that the expression of CPT1a and -b was not significantly different between wt and staggerer littermate mice. However, we observed a significant ~1.5-fold increase in MCAD mRNA expression in white adipose (but not in liver or skeletal muscle) tissue (Table 1). It should be noted that it has been reported that “increased flux of fatty acids through the β-oxidation pathway does not necessarily require marked changes in expression of genes involved in metabolism” (42).

β2-Adrenergic Receptor mRNA Expression Is Significantly Increased in Brown Adipose Tissue—We also explored whether the reduced adiposity involved sympathetic regulation of lipid metabolism. We examined the expression of the β1-, β2-, and β3-adrenergic receptors (β1:−AR/ADRB1–3) in liver, white and brown adipose, and skeletal muscle tissue. We did not observe any significant increases in the expression of these adrenergic receptors in liver, white adipose, and muscle tissue (Fig. 7, A–C). However, we did observe a small but significant

FIGURE 5. Binding and transactivation of SREBP1c gene promoter by RORα. A and B, diagrammatic representation of nuclear receptor response elements on mouse SREBP1c promoter investigated in this study. C, the recruitment of RORα onto SREBP1c nuclear receptor response elements in muscle C2C12 cells by ChIP assay (representative assay). Results are expressed as the mean ± S.D. D, transient transfection of mouse pGL3-SREBP1c gene reporter with pSG5-RORα1 or pSG5-RORα4 or pSG5 vector in COS-1 cells. Results are expressed as the mean -fold activation ± S.E. (n = 3). *, p < 0.05. E, transient transfection of mouse pGL3-SREBP1c gene reporter with pSG5-RORα1 or pSG5-RORα4 or pSG5 vector in skeletal muscle C2C12 cells. F, transient transfection of mouse pTK-LUC-SREBP1c (−1342/-1158) reporter with pSG5-RORα1 or pSG5-RORα4 or pSG5 vector in skeletal muscle C2C12 cells. Results are expressed as the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01. The -fold activity of pGL3 basic or pTK-LUC cotransfected with pSG5 is arbitrarily set to 1.

present in our SREBP-1c reporter. Hence, we subsequently cloned a single copy of the promoter region between −1342 and −1158 into the TK-luc basal promoter to examine whether this fragment supported RORα-mediated transcription. Interestingly, this region is selectively trans-activated by RORα. In summary, this demonstrated RORα was directly recruited to, and modulated the activity of the SREBP-1c promoter.

PGC-1 and Lipin1 mRNA Expression Are Significantly Increased in the Liver and White and Brown Adipose Tissue of sg/sg Mice—We further explored underlying changes in gene expression (in addition to decreased SREBP-1c) that may account for reduced adiposity. Hence, we utilized candidate-based expression profiling to examine whether the expression of genes that regulate fatty acid oxidation (and oxidative metabolism) are modulated in sg/sg mice.

PGC-1α and -1β are important regulators of fatty acid oxidation and oxidative metabolism (38). We observed that the expression of the mRNA encoding PGC-1α is increased in liver and white adipose tissue (but not in brown adipose tissue or skeletal muscle) of fasted sg/sg mice, relative to littermate control wt mice (Fig. 6, A, D, G, and J). However, the increase in white adipose tissue just failed to attain statistical significance (p = 0.051). PGC-1α induces the gluconeogenic program in the liver (38). Therefore, we investigated the levels of PEPCk (as an indicator of functional PGC-1 induction). In agreement with elevated PGC-1α expression, we observed significantly elevated levels of PEPCk mRNA in the liver of fasted sg/sg mice (relative to the littermate control, wt mice) (data not shown).

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decrease in β7-AR (ADRB3) expression in the white adipose tissue from sg/sg mice, relative to wt littermates. We observed a significant (3- to 4-fold) increase in β3-AR (ADRB2) expression in the brown adipose tissue from sg/sg mice.

### Table 1

| Tissue            | Gene   | WT       | sg/sg     | Relative Expression |
|-------------------|--------|----------|-----------|---------------------|
| Liver             | CPT1a  | 1.98 ± 0.0001 | 2.13 ± 0.0002 | 1.09 ± 0.02       |
|                   | MCAD   | 2.97 ± 0.0002 | 3.04 ± 0.0003 | 1.02 ± 0.02       |
| White adipose     | CPT1a  | 6.22 ± 0.0005 | 5.04 ± 0.0006 | 1.23 ± 0.02       |
|                   | MCAD   | 1.37 ± 0.0004 | 1.94 ± 0.0004 | 1.42 ± 0.02       |
| Skeletal muscle   | CPT1b  | 4.65 ± 0.0005 | 4.20 ± 0.0005 | 1.09 ± 0.02       |
|                   | MCAD   | 2.52 ± 0.0005 | 2.29 ± 0.0005 | 1.10 ± 0.02       |

*p < 0.05.

**Figure 6.** Analysis of PGC-1α, PGC-1β, and lipin1 mRNA expression in liver, epididymal (white) fat pad, brown (interscapular) fat, and quadriceps muscle. Quantitative PCR of PGC-1α in liver (A), white adipose (D), brown adipose (G), and skeletal muscle (J) tissue, respectively, and PGC-1β mRNA levels in liver (B), white adipose (E), brown adipose (H), and skeletal muscle (K) tissue, respectively, of male wt and sg/sg mice. Lipin1 mRNA levels in liver (C), white adipose (F), brown adipose (I), and skeletal muscle (L) tissue, respectively, of male wt and sg/sg mice. Relative mRNA expression is normalized against 18 S rRNA. Results are expressed as the mean ± S.E. (n = 7–8). *, p < 0.05 and **, p < 0.01.

**Figure 7.** Analysis of β-ARs and NR4A mRNA expression in male wt and sg/sg mice. Quantitative PCR of ADRB1 (A), ADRB2 (B), ADRB3 (C), NUR77 (D), NURR1 (E), and NOR-1 (F) in liver, epididymal (white) and interscapular (brown) fat pads, and quadriceps muscle. Relative mRNA expression is normalized against 18 S rRNA. Results are expressed as the mean ± S.E. (n = 7–8). **, p < 0.01.
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FIGURE 8. Examination of gene expression regulating lipid mobilization and energy expenditure. Expression profiling of the mRNAs encoding ATGL, HSL, LPL, LIPC, and UCP1–3 in liver, white and brown adipose, and skeletal muscle. Quantitative PCR of mRNA levels of LPL (A), ATGL (B), HSL (C), and LIPC (D), in liver, epididymal fat pad, and quadriiceps muscle of male wt and sg/sg mice (n = 7–8), and UCP1 (E), UCP2 (F), and UCP3 (G) in liver, epididymal (white) and interscapular (brown) fat pads, and quadriiceps muscle of male wt and sg/sg mice (n = 7–8). Relative mRNA expression is normalized against 18S rRNA. Results are expressed as the mean ± S.E. (n = 7–8).

Furthermore, adrenergic signaling has been demonstrated to induce the expression of the nuclear receptor subfamily 4, group A (NR4A) subgroup of nuclear receptors that regulate metabolism (18, 19, 43–45). Hence, we examined the expression of these NRs. We did not observe significant changes in the expression of NR4A1–3 in the liver, adipose, and skeletal muscle tissues (Fig. 7, D–F). However, we observed 2- to 3-fold increases in NOR-1 in white and brown adipose in staggerer mice, but these changes did not attain significance (p = 0.12 and 0.2, respectively).

The increased β3-AR (and NOR-1) expression in the brown adipose tissue from staggerer mice is consistent with the 30–70% higher metabolic rate (depending on temperature) and the increased levels of endogenous norepinephrine in (cold reared) staggerer mice relative to control mice (15, 46).

Reduced Adiposity Does Not Involve Changes in Gene Expression (Including Nuclear Hormone Receptors) That Control Lipid Mobilization in Liver, Adipose, and Muscle Tissue—Interestingly, the decreased adipose mass and adipocyte cell size did not correlate with increased lipid mobilization. Staggerer mice, expressed similar levels of lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), hormone-sensitive, and lipase C (hepatic lipase, LIPC) relative to wild littermate mice (Figs. 8, A–D) in liver, white adipose, and skeletal muscle tissue. In addition, we examined the expression of the uncoupling protein genes (UCP1–3) that regulate energy expenditure (and lipid utilization). Expression in the liver, adipose, and skeletal muscle tissue from wt and sg/sg mice was essentially similar (Fig. 8, E–G). However, the expression of UCP-3 (involved in preferential free fatty acid utilization in rodent and human skeletal muscle (47, 48)) mRNA was increased (but did not attain significance, p = 0.2) in skeletal muscle from sg/sg mice (Fig. 8F). Moreover, increased expression (that did not attain significance, p = 0.09) of UCP-1 in brown adipose was also observed, and this correlated with the increases in NOR-1 expression in this tissue (Fig. 8G). Sheila Collins and colleagues very recently reported the NOR-1-dependent induction of UCP-1 (46).

Finally, we examined a panel of nuclear receptors that are known to function as master regulators of lipid and cholesterol homeostasis. The expression of LXRα and -β, estrogen-related receptor (ERR) α/γ, and peroxisome proliferator-activated receptor (PPAR) α, β/δ, and γ was similar in liver and adipose tissue from sg/sg and wt mice (data not shown).

In summary, the staggerer phenotype is characterized by underlying changes in gene expression that regulate lipogenic and oxidative metabolism. However, we did not observe changes in expression of genes that control lipid mobilization and energy expenditure. Neither did we observe changes in the expression of nuclear receptors (NRs) that are master regulators of lipid homeostasis.

Staggerer Mice Are Resistant to Diet-induced Obesity—We subsequently examined the physiological response of staggerer mice to an energy dense (high fat) diet.

Male and female wild-type and sg/sg mice were placed on standard and high fat diets for a 10-week period. Interestingly, male and female sg/sg mice are completely resistant to diet-induced weight gain and obesity. For example, no significant differences in the weight of male and female sg/sg mice were observed over a 10-week period on a regular chow diet (<10% of total calories from fat) compared with those on a high fat diet (~40% of energy from fat). In contrast, the male and female wt mice, experience increases of ~17% (Fig. 9, A and C) and 22% (Fig. 9B) in weight, respectively, after 10 weeks on the high fat relative to the regular diet.

This was underscored by increased adiposity (Fig. 9D) and significantly increased amounts of epididymal and inguinal white adipose (Fig. 9E) in the wt, relative to male sg/sg mice (on the standard diet). Furthermore, this difference in the epididymal and inguinal fat depots was highly significant and markedly amplified in the wt mice after 10 weeks on the high fat diet (Fig. 9E). In addition, significantly increased amounts of brown adipose (interscapular fat) in wt mice (relative to the sg/sg) was also observed in male mice on the high fat diet (Fig. 9G). This correlates with increased adipocyte cell size in brown adipose from wt versus sg/sg mice (Fig. 9H).

Similarly, increased ovarian and inguinal fat depots in wt mice (relative to the sg/sg) were also observed in females on the high fat diet (Fig. 9F). In contrast, no significant differences in corrected heart and liver tissue weights (from male wt and sg/sg mice) were observed (Fig. 9E) on the chow (standard) and/or high fat diets. Notably, analysis of sg/sg mice revealed the distinct lack of fat accumulation in the gonadal and inguinal adipose in the sg/sg mice (relative to the wild-type mice) on the high fat diet. Moreover, the white adipose fat pad sizes in sg/sg mice were similar on the regular chow and high fat diets (Fig. 9E).

Interestingly, in the context of where the fat was being deposited, we also analyzed hepatic triglyceride levels from male and female wt and sg/sg mice on the high fat diet. We report that the triglyceride concentrations (milligrams/g liver wet weight) are
RORα Regulates Adiposity

Two isoforms of RORα are expressed in mice (α1 and α4) (49). Using quantitative PCR analysis, we showed that the mRNA encoding RORα1 is the predominantly expressed isoform in liver, white adipose, and skeletal muscle tissue. However, expression of both the RORα1 and -α4 transcripts was diminished in sg/sg mice.

Our results indicated that sg/sg mice have a complex phenotype with lower body weight, reduced adiposity, decreased plasma cholesterol, non-esterified fatty acid, and reduced triglycerides (in the serum and liver) and are refractory to diet-induced obesity.

As discussed, Staels et al. (11) have reported an association between decreased HDL-c and intestinal ApoAI production in sg/sg mice. Here we demonstrate that these mice also exhibit decreased expression of the reverse cholesterol transporters, ABCA1, and ABCA8/G1 in the liver. Studies that utilize a liver-specific knock-out of ABCA1 have established a crucial role for this transporter in HDL biosynthesis (51). Hence, sg/sg mice exhibit impaired HDL biosynthesis as a result of decreased expression of ABCA1.

We have also observed a reduction in liver (and plasma) triglycerides in sg/sg mice compared with normal wt littermates.

**FIGURE 9. Tissue and body weight gain in wt and sg/sg mice on high fat diet.** Comparison of body weight gain of male (A) and female (B) wt and sg/sg mice on normal chow diet (Chow) and high fat diet (HFD) (n = 6–20). C, photograph showing reduced body size in male sg/sg mice on HFD; significantly lower in the hepatic tissue from male and female sg/sg mice, relative to wild-type littermates (Fig. 9, I and J).

Furthermore, to examine whether resistance to diet-induced obesity, and the distinct lack of fat accumulation in the adipose (and liver) from sg/sg mice (on a high fat diet) involved hyperphagia, we measured food intake. Guastavino et al. (16) demonstrated that staggerer mice are hyperphagic on a regular chow diet. We measured the food intake of male sg/sg and wt littermates on the high fat diet, and in concordance with the study above, but surprisingly, we observed that staggerer mice consumed double the amount of food relative to their wild-type littermates, and this translated to >3-fold increase in food intake per gram of body weight (milligrams/gram of animal) (see Fig. 9K). Moreover, this correlated with significantly decreased leptin expression in white adipose tissue (Fig. 9L).

In summary, analysis of sg/sg mice revealed the striking lack of lipid accumulation in the adipose (and liver) from the sg/sg mice (relative to the wild-type mice) on the high fat diet, despite hyperphagia.

**DISCUSSION**

Two isoforms of RORα are expressed in mice (α1 and α4) (49). Using quantitative PCR analysis, we showed that the mRNA encoding RORα1 is the predominantly expressed isoform in liver, white adipose, and skeletal muscle tissue. However, expression of both the RORα1 and -α4 transcripts was diminished in sg/sg mice.

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The mechanism involves suppression of the transcriptional regulator, SREBP-1c, and suppression of the lipogenic gene program is responsible for this phenotype. For example, sg/sg mice expressed significantly reduced levels of the mRNAs encoding SREBP1c, FAS, and ABCG1. SREBP-1 is a critical transcription regulator that controls the expression of genes involved in fatty acid biosynthesis and the nutrition-dependent induction of lipogenesis (52). SREBP-1c directly regulates the lipogenic enzymes, including FAS, by selectively binding to the target promoters, however, FAS can also be regulated by SREBP-1c-independent mechanisms that involve trans-activation by LXR (53). Mice treated with the LXR agonist, T0901370, display hypertriglyceridemia and elevated hepatic expression of the mRNAs encoding SREBP-1c and FAS. Analogously, suppression of FAS lowers serum triacylglycerol levels (54). Our observation in the sg/sg mice (of reduced SREBP-1c and FAS expression) is concordant with these reports. In addition, we observed significantly decreased hepatic ABCG1 expression. This is entirely consistent with a recent study that demonstrated ablation of this ATP-binding cholesterol transporter reduced body weight gain, adiposity (and adipocyte size), and serum triglycerides, all features of the staggerer line (28).

We investigated the link between dysfunctional RORα and decreased lipogenic gene expression and reduced triglycerides. SREBP-1c (and its downstream target FAS) are well-characterized LXR target genes. LXR regulates hepatic lipogenesis. Moreover, it modulates ChREBP expression, a direct regulator of lipid synthesis. ChREBP consequently regulates FAS and the enzymes that channel the glycolytic end-products into lipogenesis (for example, pyruvate kinase-liver type) (29, 30). Hence, LXR (denoted master lipogenic factor) and ChREBP are important transcriptional regulators of hepatic lipogenesis (in concert with SREBP-1c). Consequently, we examined the expression of LXR (LXRα and LXRβ) and ChREBP in sg/sg mice. Interestingly, we did not observe any significant changes in the expression of these critical regulators, in the hepatic tissue of staggerer, relative to wild-type mice (Fig. 4, F–H). This suggested that the aberrant triglyceride levels in the liver and plasma were primarily the result of attenuated SREBP-1c expression. Furthermore, RORα transfection and ChIP studies suggested that dysfunctional RORα expression is involved in attenuated SREBP-1c expression.

The increased expression of PGC-1α/β and lipin1 (regulators of oxidative metabolism) in the liver and white and brown adipose tissue of the sg/sg mice is consistent with the reduced adiposity of the sg/sg mice reported here. For example, in diet and genetic mediated mouse models of obesity (55), the expression of PGC-1α, a cellular regulator of oxidative metabolism and energy production (Ref. 56 and references therein) is diminished. Likewise, lipin1 expression is decreased in obesity and elevated by weight reduction (57).

In addition, further analysis of brown adipose tissue revealed significantly increased β3-AR mRNA expression in brown adipose tissue from staggerer mice (relative to the wt littermate). This was coupled to 2- to 3-fold increases in NOR-1 and UCP-1 in this tissue. These observations are consistent with previous reports that demonstrated 30–70% higher metabolic rate (depending on temperature) and higher endogenous levels of norepinephrine in (cold reared) sg/sg mice relative to control mice (15). However, norepinephrine treatment results in similar increases in oxygen consumption in sg/sg and control mice. Furthermore, adrenergic signaling regulates lipolysis (58) and has been demonstrated to induce the expression of the NR4A subgroup of nuclear receptors that regulate metabolism in muscle, adipose, and liver (18, 19, 43–46, 58–60).

Subsequently, we investigated the response of the sg/sg mice to a metabolic challenge involving an energy dense (high fat) feeding regime. Both male and female sg/sg mice were completely refractory to fat accumulation and diet-induced obesity. This was underscored by dramatic increases in white and brown adipose depots, and hepatic triglycerides in wild-type mice, but not in sg/sg mice after a period of 10 weeks on a high fat diet. This demonstrates RORα operates as an important modulator of lipid homeostasis in liver and white and brown adipose tissue.

Our report demonstrates the lean phenotype in sg/sg mice is characterized by significantly decreased SREBP-1c (and lipogenic gene) expression and increased expression of PGC-1α/β, lipin1, and β3-AR expression in liver, muscle, and (white and brown) adipose tissue. This is concordant with the previously reported increased metabolic rate/O2 consumption and higher endogenous epinephrine levels in brown adipose from staggerer mice. These observations, coupled to increased expression of genes controlling oxidative metabolism and decreased expression of genes involved in lipid deposition, provide molecular explanations for reduced adiposity and hepatic triglycerides in staggerer mice. These factors provide molecular evidence for the resistance to diet-induced obesity, despite hyperphagia (which correlated to reduced leptin expression in adipose tissue) (17, 50, 61).

In conclusion, we suggest this orphan NR operates as an important modulator of the equilibrium between anabolic and catabolic lipid metabolism in hepatic and peripheral metabolic tissues. The link between dysfunctional RORα and protection against obesity suggests this orphan NR may have potential utility in the treatment of metabolic disease. The challenge for the future is the identification of organ-specific selective modulators/antagonists.

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