Identification and validation of the pathways and functions regulated by the orphan nuclear receptor, ROR alpha1, in skeletal muscle


The University of Queensland, Institute for Molecular Bioscience, Brisbane, Queensland, 4072, Australia

Received December 2, 2009; Revised February 25, 2010; Accepted March 2, 2010

ABSTRACT

The retinoic acid receptor-related orphan receptor (ROR) alpha has been demonstrated to regulate lipid metabolism. We were interested in the RORα1 dependent physiological functions in skeletal muscle. This major mass organ accounts for ~40% of the total body mass and significant levels of lipid catabolism, glucose disposal and energy expenditure. We utilized the strategy of targeted muscle-specific expression of a truncated (dominant negative) RORα1ΔDE in transgenic mice to investigate RORα1 signaling in this tissue. Expression profiling and pathway analysis indicated that RORα1 influenced genes involved in: (i) lipid and carbohydrate metabolism, cardiovascular and metabolic disease; (ii) LXR nuclear receptor signaling and (iii) Akt and AMPK signaling. This analysis was validated by quantitative PCR analysis using TaqMan low-density arrays, coupled to statistical analysis (with Empirical Bayes and Benjamini–Hochberg). Moreover, westerns and metabolic profiling were utilized to validate the genes, proteins and pathways (lipogenic, Akt, AMPK and fatty acid oxidation) involved in the regulation of metabolism by RORα1. The identified genes and pathways were in concordance with the demonstration of hyperglycemia, glucose intolerance, attenuated insulin-stimulated phosphorylation of Akt and impaired glucose uptake in the transgenic heterozygous Tg-RORα1ΔDE animals. In conclusion, we propose that RORα1 is involved in regulating the Akt2-AMPK signaling pathways in the context of lipid homeostasis in skeletal muscle.

INTRODUCTION

Retinoic acid receptor related orphan receptor alpha (RORα) is an orphan member of the nuclear receptor superfamily of transcription factors. Several in vitro and in vivo studies on RORα action and function (1–5) have suggested the involvement of this orphan nuclear receptor in lipid homeostasis (6) and hepatic phase I/II metabolism (7). RORα can be detected in many metabolic tissues including liver, kidney, adipose tissue and is highly expressed in skeletal muscle. In mice, RORα deficiency leads to profound metabolic disturbances. The homozygous staggerer (sg/sg) mice have a global RORα defect that results in decreased and dysfunctional expression of both mouse isoforms of RORα (1 and 4). These mice display hypoalphalipoproteinemia, dyslipidemia (decreased serum triglycerides and HDL-cholesterol) (5), susceptibility to atherosclerosis (2) and reduced adiposity and resistance to diet-induced obesity (8). The complex phenotype of the staggerer mice has been demonstrated to involve underlying changes in the expression of genes involved in fatty acid homeostasis, i.e. Apo-lipoprotein A1 (ApoA1) (5), Apo-lipoprotein C3 (ApoCIII) (1), sterol regulatory element-binding protein 1c (SREBP-1c), ATP-binding cassette transporter-binding proteins A1 and G1 (ABCA1 and G1), peroxisome proliferator-activated receptor gamma co-activator alpha/beta (PGC-1α/β), lipin1 and beta2-adrenergic receptor (8).

In the context of whole body metabolism, skeletal muscle has a vital contribution to the maintenance of energy balance. It is a major mass peripheral tissue with high energy demands. Consequently, multiple metabolic pathways converge in this tissue involving the utilization of both lipid and carbohydrate substrates. Skeletal muscle is considered a major site of glucose disposal and perturbation of insulin-mediated glucose uptake in this tissue is an important factor in the development of type 2 diabetes.
In addition, the development of insulin resistance in skeletal muscle has been associated with increased intramuscular triglyceride accumulation, which can attenuate several insulin signaling pathways (9). As type 2 diabetes and associated complications are health issues that have global significance, it is therefore of considerable interest to investigate the regulatory machinery responsible for maintaining tight metabolic control in this tissue.

Previously, our analysis of skeletal muscle from staggering mice (8) identified differential expression of a number of genes involved in fatty acid homeostasis. In this study, we aimed to investigate the role of RORα in skeletal muscle, without the complex interactions that result from the global defect. Therefore, we generated a transgenic mouse that overexpressed truncated human RORz1ΔDE (lacking the ligand-binding domain) in skeletal muscle to investigate the contribution of this energy demanding tissue to the RORα phenotype. We used a three-pronged genomic approach, utilizing lumina expression profiling, Ingenuity function and pathway analysis, and validation by rigorous quantitative PCR (qPCR) analysis on the TaqMan® low density array (TLDAs) platform. This demonstrated that RORα has an important role in the regulation of carbohydrate and lipid metabolism in skeletal muscle. In particular, this animal model demonstrated that RORz1 also plays a critical role in skeletal muscle insulin signaling and glucose tolerance, via modulation of Akt2 and adenosine monophosphate kinase (AMPK) expression and activity. This correlated with increased expression of phospho ACC and genes involved in the regulation of fatty acid oxidation. In conclusion, we propose that in skeletal muscle RORα is an important metabolic regulator, contributing to both glucose and fatty acid homeostasis.

MATERIALS AND METHODS

Production of transgenic-mouse

The transgenic vector construct encodes a truncated version of human RORz1 (RORz1ΔDE). Amino acids 1–235 are present but the entire E region and part of the hinge/D region have been removed as previously described (3). To confer muscle specificity, the vector was placed under the control of the full-length human skeletal alpha-actin (HSA) promoter as described previously (10–12). Zygotes were generated by pronuclear microinjection of the transgene into oocytes from hybrid female donors (C57BL/6J X CBA) as described (13). Screening of founder mice and their offspring for stable germ line transmission was performed by real time PCR. The sequences of the specific mouse RORz and human RORz primers were as follows—hRORz: CAATGCCACCTACTCCTGTCC and CTACGGCAAGGCATTCTGT AAT, mRORz: CAATGCCACCTACTCTGTCC and GCGAGCATTCTGCAGC. Transgene copy number was determined using real time PCR as described (14). Two founders were selected for analysis, each of which carried two copies of the transgene. Both lines were then backcrossed with C57BL/6J mice for a minimum of five generations. Experiments were performed on transgenic mice from the fifth generation onward, relative to wt littermates; therefore the genetic background of the animals in this study is >98.6% C57Bl/6J. The initial characterization was performed on both transgenic lines. No differences were observed in either phenotype or metabolic gene expression between the two lines, therefore we continued investigation on one line only.

Animal procedures

The mice were housed in the QBP vivarium (University of Queensland, St. Lucia, Queensland, Australia) with 12 h light–dark cycle and fed a standard diet containing 4.6% total fat (from Specialty Feeds, Glen Forrest, Western Australia). For the diet-induced obesity experiments, mice were transferred to a high-fat diet containing 34.9% fat (D12492, Research Diets, New Brunswick, NJ) for 4 weeks of age onward. Experimental animals were weighed weekly up to 14 weeks of age. Mice were fasted overnight by transferring to a new food-free holding cage with unrestricted access to water, prior to all experimental procedures. Care was taken to euthanize all animals at 9 am (i.e. 3 h into the light cycle, and at similar times), and excised tissues were immediately frozen in liquid nitrogen and then stored at −80°C. All aspects of animal experimentation were approved by The University of Queensland Animal Ethics Committee.

Microarray analysis

It is completely described in Supplementary Figure S1.

RNA extraction, cDNA synthesis and qPCR TLDAs analysis

Total RNA extraction and cDNA synthesis were performed as described previously (4). We utilized custom designed ABI microfluidic TLDAs to analyze the expression of genes involved in metabolism (lipid, carbohydrate and energy homeostasis). Three control genes were utilized, including the mandatory control (18S rRNA) and four other controls: Gapdh, GusB, Hprt1 and 36B4. These controls span the relative abundance/Ct range of the genes on the card, and three (18S rRNA, GAPDH and 36b4) are approved real time PCR controls for NURSA supported nuclear receptor studies (15,16). The TLDAs were analyzed as described in Myers et al. (17). Briefly, significant changes in expression relative to wt littermate mice were analyzed using the ABI/integromics ‘StatMiner’ software package. Differentially expressed genes were identified by linear models (contained in the LIMMA package for bioconductor R embedded in StatMiner). Significance was assigned by the application of the Empirical Bayes statistic. B values represent the empirical bayes log odds of differential expression, and the t-value is the empirical bayes moderated t-statistic. Subsequently, we applied a more stringent/conservative data filtering (Benjamini–Hochberg) to control for false discovery rate, correct P values and further refine the subset of differentially expressed genes.
Primers and qPCR

Relative expression of genes was determined using the ABI 7500 real time PCR System (ABI, Singapore) as previously described (3). Primers for LXRα, GAAATGCCA GGAGTGTGCAC and GATCTGTTCTTCTGACAGC (for ACACA); Akt1, GGCTGGCTGCAAAACG and GAC TCTCGCTGATCCACATCCT; Akt3, CCTTCCAGAC AAAAAACGGTTT and CGCTCTTGACAAATGG AAA; GLUT1, TACGCTGAGGCGGTAGCT and A ATGGGCCGATCCTAAAATGG.

Protein extraction

Total soluble protein was extracted from skeletal muscle (quadriceps) by the addition of lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100 and 5 mM EDTA) containing protease and phosphatase ‘cocktail’ inhibitors (Roche diagnostics GmbH, Mannheim, Germany). Lysates were passed through a 26-gauge needle and centrifuged at 10,000g for 20 min. The supernatant was collected and total protein concentration was determined by the bicinchoninic acid (BCA assay kit), as outlined by manufacturer’s instructions (Pierce Biotechnology Inc., Rockford, IL, USA).

Glucose and insulin tolerance tests and glucose uptake

Basal glucose measurements were obtained from the tail blood of overnight fasted animals (14–16 weeks of age). Mice were then administered a dose of either glucose solution (2 g/kg) or insulin (0.5 U/kg) by intraperitoneal injection. Blood glucose measurements were obtained at 10 or 15 min intervals for up to 90 min following challenge using the Accu-Chek Performa blood glucose testing system (Roche Diagnostics Australia, Castle Hill, NSW). Plasma insulin measurements were obtained using the insulin (mouse) Ultrasensitive EIA (ALPCO Diagnostics, Salem NH). Insulin-stimulated glucose uptake was performed on skeletal muscle (extensor digitorum longus, EDL) dissected from anesthetized mice and incubated in an essential buffer (Krebs–Henseleit, pH 7.4) and the assay was performed as described previously (18,19).

Western blot analysis

Total soluble protein from the quadriceps of transgenic and their littermate wild-type mice were resolved on a 10% SDS–PAGE gel and transferred to a PVDF (Millipore Corporation, Billerica, MA, USA). The membranes were blocked for 1 h in 5% BSA in TBS–TWEEN 20, followed by an overnight incubation with primary antibody. The following antibodies were purchased from Cell Signaling Technology, Danvers, MA and used at 1:1000 dilution: AMPKα (#2532), pAMPK (Thr172) (#2535), Akt (#9272), pAKT (ser473) (#4058), ACC (#3662) and pACC(Ser79) (#3661). Anti-GAPDH (1:10000) was from R&D Systems, Minneapolis, MN. Following 3 x 10 min washes, the membrane was incubated with anti-rabbit horseradish peroxidase (HRP) (1:10000) for 1 h. Immunoreactive signals were detected using enhanced chemiluminescence Super Signal West Pico Substrate (Pierce) and visualized by autoradiography on an X-OMAT film developer (Kodak).

Chromatin immunoprecipitation analysis

C2C12 cells were differentiated for 4 days. Cells were harvested and subsequently washed twice in ice cold PBS and cross-linked in 1% formaldehyde solution. Chromatin immunoprecipitation (ChiP) was performed as described by Pearen et al. (20) using anti-RORα (Santa Cruz anti-RORα sc-6062) and IgG (Santa Cruz, CA). The following qPCR ChiP primers were used: RORE Site 1 F—GCATGTGCTGCAAACATTCAG (−2897 to −2876), R—CTACACAGGGTCGTGCGCCA (−2866 to −2846). RORE Site 2 F—GAACATGAAGATTTGAA CTIG (−2705 to −2684), R—CTGACCCAGAAACTCTCA (−2668 to −2649). RORE Site 3 F—CTGGGTCGATGAGTGCAAGCC (−2657 to 2634), R—TAA GCATTGGAGGTGAACCTGTAA (−2631 to 2606). Downstream Negative control F—GTTCCAAGTGTGA GAACCGC (−278 to −258), R—CCGCAATCAAGGG CCTT (−195 to −177).

Statistical analysis

The TLDa gene expression data were analyzed as described above. All other results were analyzed (and significance assigned) using a t-test, or ANOVA in the Graphpad Prism 4 software, unless otherwise indicated.

RESULTS

Overexpression of truncated RORαDE (lacking the ligand-binding domain) in skeletal muscle

As discussed, RORα has been demonstrated to regulate fat metabolism in several tissues. We were interested in identifying and validating the (physiologically relevant) in vivo functional role(s) and pathway(s) regulated by RORα action in skeletal muscle. This major mass peripheral tissue accounts for ~40% of the total body mass, significant levels of fatty acid oxidation, glucose disposal and energy demand. We utilized the approach of attenuating RORα signaling, by the targeted skeletal muscle-specific expression of a truncated RORαDE (lacking the ligand-binding domain) in transgenic mice, to examine RORα action in this tissue. This approach was utilized in the absence of the availability of a reproducible and robust, native or synthetic agonist for the modulation of this orphan nuclear receptor.

We produced transgenic mice (by pronuclear injection) that selectively express a transgene encoding truncated human RORα1 in skeletal muscle [under the control of the human skeletal alpha actin promoter (10,11)]. The transgene (RORα1DE) lacks the ligand-binding domain and part of the hinge region, and encoded amino acids 1–235 (6). McBroom et al. (21) reported that deletion of this segment preserved DNA recognition and binding, suppressed trans-activation and operated in a dominant negative manner. In addition, Hamilton et al. (22) reported the staggerer mutation is located in a similar position, and produces a non-functional ligand-binding
domain. Moreover, we previously demonstrated in an in vitro myogenic cell culture model that this construct attenuated RORx mediated trans-activation, and ectopic expression suppressed endogenous RORx mRNA expression (3).

We observed that the heterozygous transgenic mice predominantly (and abundantly) expressed the ectopic transcript (transgene) in skeletal muscle relative to other organ/tissues (cerebellum, spleen, heart, white adipose, liver and pancreas; Figure 1A and B). Heart and brain expressed <2% of the ectopic transcript, relative to expression in skeletal muscle. This was consistent with several other studies that utilized this promoter in transgenic models (10,12,23). Quantitative real-time PCR (q-PCR) analysis of mRNA expression demonstrated that the ectopic truncated RORx transcript was expressed between 3- and 4-fold higher than the endogenous RORx transcript in the muscle of transgenic mice (Figure 1A). As expected, the ectopic RORx transcript was not detectable in wt littermates (data not shown). Concordantly, expression analysis demonstrated that total (endogenous plus ectopic) RORx transcript expression increased ~3-fold in the muscle of transgenic, relative to wt littermate mice (Figure 1C). In this background we observed a decrease (that did not attain significance) in the levels of total endogenous RORx1 and 4 mRNA expression in the muscle of transgenic relative to wt littermate mice (Figure 1D). Surprisingly, isoform-specific q-PCR analysis of RORx1 and RORx4, revealed significant suppression of RORx1 (but not RORx4) mRNA expression (Figure 1E and F).

Expression profiling of skeletal muscle from the RORx1ΔDE mice: identification of functions and pathways

To rigorously identify the in vivo functional role(s), and pathway(s) regulated by RORx action in skeletal muscle, we carried out expression profiling coupled to functional and signaling characterization by Ingenuity pathway analysis (Supplementary Figures S2–S5). Using a P value cut-off of $P < 0.05$ and a fold change cut off of

---

**Figure 1.** (A) qPCR of the ectopic transgene (hRORx1ΔDE) and endogenous RORx expression in various tissue/organ in heterozygous transgenic mice. Relative mRNA expression is normalized against 18S mRNA ($n = 6$/group, mean ± SEM). **(B)** qPCR of the ectopic transgene (hRORx1ΔDE) in pancreas versus skeletal muscle from wild-type and heterozygous transgenic mice. Relative mRNA expression is normalized against 18S mRNA ($n = 6$/group). **(C)** qPCR of total RORx (ectopic and endogenous) in skeletal muscle of wild-type and transgenic mice. Relative fold change of (mRNA expression) is normalized against 18S mRNA ($n = 6$/group, mean ± SEM, ***$P < 0.001$). **(D)** qPCR of endogenous RORx1 and 4, (E) endogenous RORx1ΔDE and (F) exogenous RORx4 mRNA expression in skeletal muscle of wild-type and transgenic mice. Relative fold change of (mRNA expression) is normalized against 18S mRNA ($n = 6$/group, mean ± SEM, $**P < 0.001$). **(G)** qPCR of ApoA5 mRNA expression in skeletal muscle of wild-type and transgenic mice. Relative fold change of (mRNA expression) is normalized against 18S mRNA ($n = 6$/group, mean ± SEM, ***$P < 0.001$).
1.3, the top 50 annotated genes that were differentially up- and down-regulated in a significant manner are shown in Tables 1 and 2, respectively [the complete list of genes (including the non-annotated genes) is shown in Supplementary Figure S2]. In concordance with the overexpression of a dominant negative, the expression profiling, Genespring and Ingenuity analysis identifies a majority of genes in the down regulated category, and the bulk of highly ranked functions and pathways were attenuated/suppressed (This is evident in the complete list of genes in Supplementary Figure S2 and in the graphical representations of the data in Supplementary Figures S3–S5).

Interrogation of differentially expressed genes on the Ingenuity platform identified that a subset of differentially expressed genes in Tg-RORα1ΔE mice were involved in/associated with lipid metabolism, small molecule transport and biochemistry, cardiovascular and metabolic disease, carbohydrate metabolism, endocrine system disorders etc (see Supplementary Figure S4), in concordance with the pathophysiological role of NRs. The majority of metabolic genes are down regulated in the major functional categories of lipid and carbohydrate metabolism (Supplementary Figure S4 and S5). This is consistent with several investigations in RORα deficient mouse models (2,8). In the framework of this investigation, the primary signaling pathways regulated in skeletal muscle were the glutathione metabolism (responsible for the tight control of ROS levels, which modulate insulin sensitivity), type 2 diabetic signaling and fatty acid metabolism, carbohydrate metabolism, endocrine system disorders etc.
Expression of the truncated orphan nuclear receptor, RORα, affected the fatty acid biosynthetic pathway: decreased SREBP-1c, LXRα and downstream target genes in Tg-RORαΔDE mice

Previously we have demonstrated that staggerer mice (sg/sg), with decreased and dysfunctional RORα, affected the fatty acid biosynthetic pathway: decreased SREBP-1c, LXRα and downstream target genes in Tg-RORαΔDE mice.
The method to control *P* value false discovery rate (FDR) refined the significant subset of differentially expressed genes to SREBP-1c, FAS, SCD-1, ACSL4, CD36 and HIF1α (Figure 2A and Table 3).

Subsequently, we used (manual) qPCR to examine (and validate) the expression of several other genes in the lipogenic pathway that were identified in the illumina analysis as significant, differentially expressed targets. The Tg-RORαΔE mice also displayed significantly reduced expression of the mRNAs encoding the nuclear hormone receptor, LXRα (Figure 2B), a critical transcriptional regulator of SREBP-1c and the genetic program that regulates lipogenesis. Interestingly, the expression of both FAS and SCD-1 (responsible for the synthesis of de novo fatty acids and monounsaturated fatty acids, respectively) was also decreased. These genes are downstream targets of LXRα and SREBP1c (the master transcriptional regulators of the genetic program that...
We identified that the expression of the two mRNAs encoding tail interacting protein (Tip47) and di-acyl glycerol acetyl transferase 2 (Dgat2) were significantly reduced in the male Tg-RORα1ΔDE transgenic mice relative to wild-type littermate pairs (Figure 2C and D). In summary, we observed qPCR validation of the master transcriptional regulators of fatty acid biosynthesis (LXR and SREBP-1c) and several important downstream target genes (including FAS and SCD-1).

The observation of attenuated LXR expression and decreased downstream target gene expression was consistent with the identification of this NR signaling cascade in the pathway analysis (Supplementary Figures S4 and S5). We explored the NR signaling pathways more rigorously by performing qPCR analysis utilizing a custom-designed ABI microfluidic TLDA that encoded taqman primer sets targeting all 48 mouse NRs. The analysis revealed small, but significantly reduced (1.5–2.5-fold) expression of the mRNAs encoding the nuclear hormone receptors: LXRα, TR2, PPARδ, GR and RORγ in the Tg-RORα1ΔDE mice, relative to the wt littermates (Figure 2E, Table 3 and Supplementary Figure S7 shows the complete list of nuclear hormone receptors). Please note increased RORα expression in the Tg-RORα1ΔDE was not detected because the TLDA was mouse specific and the transgenic line expressed the truncated human transcript.

In summary, parallel analysis by illumina, ingenuity and qPCR on the TLDA platform have demonstrated that RORα in skeletal muscle leads regulates the lipogenic/fatty acid biosynthetic pathway.

Expression of truncated RORα attenuated Akt (mRNA and protein) expression in Tg-RORα1ΔDE mice

Pathway analysis revealed the involvement of RORα expression in carbohydrate metabolism and type 2 diabetic signaling, and that truncated RORα expression effected Akt2 mRNA levels (Supplementary Figures S2, S4 and S5A). This led us to further examine the expression of critical genes involved in carbohydrate metabolism using the custom-designed ABI microfluidic TLDA to perform qPCR analysis. We identified statistically significant differential expression of several important genes that control insulin signaling and glucose uptake (Table 3, Supplementary Figure S8 shows the complete list of genes analyzed) in the Tg-RORα1ΔDE mice, relative to wild-type littermates. TLDA analysis revealed the expression of the mRNAs encoding Akt2, pyruvate dehydrogenase kinase isozyme 3 and 4 (Pdk3 and 4), and several other genes were significantly reduced in the skeletal muscle of male Tg-RORα1ΔDE mice relative to wild-type littermates (see Table 3, Supplementary Figure S8). Subsequently, after correction/adjustment of P values according to the Benjamini–Hochberg FDR method, Akt2 remained the only transcript that was significantly and differentially expressed (and repressed) in the Tg-RORα1ΔDE line (see Table 3, Supplementary Figure S8 and Figure 3A). It should be noted that the data (prior to FDR filtering) has been derived from sensitive qPCR TLDA analysis from six littermate pairs of mice focused

Figure 2. Graphs showing significant changes in expression of lipogenic genes in the skeletal muscle of transgenic mice. (A) Data derived from Table 3 are expressed as fold change (log10) normalized to 18S mRNA, following application of Benjamini–Hochberg false detection rate algorithm. qPCR of (B) LXRα, (C) Tip47 and (D) Dgat2 in skeletal muscle of transgenic and wt littermate control mice. Relative fold change is normalized against 18S mRNA (n = 6/group, mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001). (E) Graphs showing significant changes in expression of the entire NR gene superfamily in the skeletal muscle of transgenic mice. Data derived from Table 3 and S7 are expressed as fold change (log10) after normalization against the median of three genorm-selected controls GAPDH, gusB and Hprt1 following application of Benjamini–Hochberg false detection rate algorithm.

modulates lipogenesis). Moreover, these changes in gene expression were not observed in either liver or adipose tissue (data not shown).

In addition, we analyzed several genes that are involved in intramuscular triglyceride (IMTG) accumulation.
concentration at time = 0 (I) control mice (glucose concentrations measured at various times after IP administration of glucose (t) (n) transgenic mice versus wt littermate controls over 14 weeks on normal chow diet (D) densitometry analysis of western blots (C) rate algorithm (derived from Table 3 and is expressed as fold change (log 10) normalized to 18S mRNA, following application of Benjamini–Hochberg false detection rate algorithm. In the skeletal muscle of transgenic mice relative wild-type mice. Data are derived from Table 3 and is expressed as fold change (log10) normalized to 18S mRNA, following application of Benjamini–Hochberg false detection rate algorithm (n = 6/group) (B) western blot analysis of Akt and pAkt in skeletal muscle of male transgenic mice and wt littermate control mice. Densitometry analysis of western blots (C) Akt and (D) pAkt (n = 3/group, mean ± SEM. *P < 0.05. **P < 0.01). (E) Body weight development in transgenic mice versus wt littermate controls over 14 weeks on normal chow diet (n = 19 male and n = 6 female, mean ± SEM. *P < 0.05). (F) Plasma glucose levels of overnight fasted male transgenic and wt littermate control mice (n = 6/group, mean ± SEM. *P < 0.05). (G) Blood glucose concentrations measured at various times after IP administration of glucose (t = 0) to overnight fasted male transgenic and wt littermate control mice (n = 7/group, mean ± SEM. *P < 0.05. **P < 0.01). (H) Blood glucose concentrations measured at various times after IP administration of insulin (t = 0) to overnight fasted male transgenic and wt littermate control mice. Data are presented as percentage of starting blood glucose concentration at time = 0 (I = 7/group, mean ± SEM).

Subsequently, we also assessed the expression of the other Akt family members. Although Akt2/PKB was significantly decreased in the skeletal muscle of male Tg-RORα1ΔDE mice relative to wild-type littermates, we observed no change in the expression of the mRNAs encoding, Akt1 or 3 (data not shown). Notably, no significant changes in gene expression were observed in either liver or adipose tissue after TLDA analysis (data not shown). In addition, we investigated whether attenuation in Akt2 mRNA expression correlated with changes in the protein levels of total Akt and phosphorylated (ser473) Akt/PKB using western analysis (Figure 3B). We observed a significant decrease in total Akt/PKB protein in the Tg-RORα1ΔDE mice relative to wild-type littermate pairs (Figure 3B and C), corresponding with the mRNA expression data. Moreover, the levels of the active phosphorylated (ser473) Akt species were also significantly decreased (Figure 3B and D). Akt/PKB is a critical target in the regulation of GLUT4-solute carrier family 2a (facilitated glucose transporter), member 4]-mediated glucose uptake. In summation, corresponding investigation by illumina, qPCR (on the TLDA platform) and western analysis have demonstrated that RORα in skeletal muscle regulates Akt2 mRNA, protein and phosphorylation. This is consistent with the high ranking of carbohydrate metabolism and type 2 diabetic signaling by the ingenuity platform.

Expression of truncated RORα in skeletal muscle induced mild hyperglycemia and glucose intolerance: attenuated insulin-mediated phosphorylation of Akt in the Tg-RORα1ΔDE mice

Suppression of (total and phospho) Akt in skeletal muscle suggests the mouse model may display increased plasma glucose and impaired glucose tolerance. We conducted several experiments to characterize the phenotypic (and metabolic) effects of aberrant RORα expression in skeletal muscle to validate the results of the illumina/ingenuity analysis.

The transgenic mice presented with no gross or histological phenotypic abnormalities, although they did appear to be slightly smaller than their wt littermates.
However, growth curve analysis from 4 to 14 weeks indicates that both male and female Tg-RORα1ΔDE mice on a regular chow diet showed no significant reductions in body weight, relative to wild-type littermate pairs (Figure 3E). All subsequent studies were performed on male mice.

We subsequently measured blood glucose and observed mild hyperglycemia in the transgenic mice, with increased fasting glucose levels in male transgenic mice relative to wild-type littermate pairs (Figure 3F). This is consistent with decreased (total and phospho) Akt (mRNA and protein) levels in skeletal muscle. We further examined systemic glucose metabolism, and performed intraperitoneal glucose and insulin tolerance tests. In the Tg-RORα1ΔDE mice, glucose clearance was significantly delayed following a glucose challenge (Figure 3G). No significant differences in plasma insulin levels between the transgenic and wt mice (on regular chow diets) were detected (data not shown). Furthermore, the glucose excursions displayed by Tg-RORα1ΔDE compared to wild-type mice during an insulin tolerance test were comparable (Figure 3H). Moreover, no differences in plasma insulin concentration were observed either at baseline or ten minutes subsequent to glucose administration (data not shown).

We subsequently examined whether skeletal muscle-specific expression of Tg-RORα1ΔDE mice played a role in insulin signaling. Protein extracts were isolated from saline and insulin injected (littermate pairs of) wt and Tg mice and subsequently analyzed by immunoblot analysis using Ab’s specific to Akt2 and phospho-Akt2.

Quantification of the western blots (Figure 4E and F) demonstrated that there were no significant differences in (basal) total Akt2 levels in between saline and insulin-treated wild-type and Tg animals. As expected, insulin treatment in wild-type mice significantly stimulated the levels of phosphoSer473Akt2 relative to saline treated mice (~3-fold Figure 4A and B). However, we observed that insulin treatment did not significantly increase phosphorylation of Akt Ser473 in the male Tg-RORα1ΔDE mice (Figure 4C and D, respectively).

Furthermore, impaired glucose tolerance and insulin stimulation of Akt phosphorylation did not involve (or result in) significant changes in GLUT2, 4 or 8 mRNA expression (determined by qPCR-microfluidic TLDA analysis, see Supplementary Figure S7). GLUT1 mRNA expression was analyzed independently by qPCR, and data presented as a footnote in Supplementary Figure S8. The expression of the mRNAs encoding Glut1, 4 and 8 was decreased by ~20%, however, after Bayes and FDR analysis, these changes did not attain significance (Supplementary Figure S8).

In the context of the hyperglycemia, impaired glucose tolerance and insulin stimulation of Akt phosphorylation, we investigated whether ex vivo glucose uptake was affected by transgenic RORΔDE expression. (Figure 4E). In wild-type type 2 (fast twitch glycolytic EDL). Figure 4E) skeletal muscle, glucose uptake was increased ~2-fold by insulin treatment. Similar results were observed in soleus muscle (data not shown).

Reduced Akt correlates with increased levels of phospho (thr172)-adenosine monophosphate kinase in Tg-RORα1ΔDE mice

Recent research has demonstrated that Akt2/PKB is a potent negative regulator of AMPK activity (Figure 6A) in murine cardiac muscle and fibroblasts (27,28). Secondly, ingenuity interrogation of the illumina data identified AMPK signaling as a significantly regulated pathway. Therefore, we undertook western analysis of the basal and phosphorylated AMPK species (Figure 6B). We detected no change in total AMPK protein levels (Figure 6B and C). However, phosphorylated T172 AMPK levels were significantly elevated in skeletal muscle from Tg-RORα1ΔDE mice (Figure 6B and C). The increased levels of phospho-AMPK are entirely consistent with reduced Akt2 activity.
We further investigated the levels of the basal and phosphorylated AMPK species AMPK in homozygous staggerer (sg/sg) mice that lack RORα in all tissues (in contrast, to the muscle-specific overexpression of the dominant negative). We detected a slight but significant \( \sim 1.5 \)-fold increase in total AMPK protein levels (Figure 6D and E) in sg/sg mice relative to wt littermates. However, the levels of phosphorylated T172 AMPK levels did not change in the skeletal muscle tissue from the sg/sg mice relative to wt littermates (Figure 6D and E). In summary, transgenic and muscle-specific overexpression of RORαΔDE leads to elevated levels of phospho-AMPK.

**Elevated phospho-AMPK in Tg-RORαΔDE mice leads to increased phosphoACC Levels, and increased PGC-1α and CPT-1b mRNA expression**

The literature reports that the positive effect of activated AMPK on fatty acid oxidation is mediated by phosphorylation of Acetyl CoA carboxylase ACC (and decreased production of malonyl CoA) (see Figure 7A).
elements in concordance with the optimal ROR Diagrammatic representation of predicted RORalpha response control was located between nucleotide positions –278/–177 (sites 1–3 for is shown on Figure 5A. The downstream negative (http://www.ensembl.org/Mus_musculus/Gene/Sequence?g=ENS sites 1–3 for is shown on Figure 5A). The downstream negative control was located between nucleotide positions –278/–177 (--1.6 kb downstream of these putative RORE sites). (C) The recruitment of RORz onto the Akt2 promoter in C2C12 myotubes by ChIP assay (representative assay) using anti-RORz (Santa Cruz anti-RORz sc-6062). Triplicate real-time PCR analysis was performed and the results are expressed, as the mean ± SD. Results are representative of two independent experiments.

AMPK activators increase the expression of PGC-1α and PPARα target genes, for example CPT-1 in skeletal muscle (29,30). Consequently, we were particularly interested to assess whether increased expression of activated AMPK resulted in the induction of phosphoACC (Ser79) and two critical genes associated with fatty acid oxidation in the skeletal muscle of the Tg-RORz1ΔDE mice (Figure 7E) in the skeletal muscle of male Tg-RORz1ΔDE mice relative to wild-type littermates. The elevated levels of pACC and PGC-1α and CPT-1 mRNA expression in the Tg mice are consistent with increased levels of pAMPK and pACC.

Expression of the truncated orphan nuclear receptor, RORz1 had minimal effects on important skeletal muscle markers of fiber type and muscle mass

We utilized qPCR-TLDA analysis to assess whether the expression of the truncated receptor affect markers of contractile function, fiber type and muscle mass (see Table 3, Supplementary Figure S9). Specifically, in this context, TLDA analysis revealed significant differential expression of the mRNAs encoding histone deacetylase 5 (HDAC5), transforming growth factor beta receptor 1 (Tgfbr1) and several other genes (including Me2a/d, Tnni2 and Acrv2A) in the skeletal muscle of male Tg-RORz1ΔDE mice relative to wild-type littermates (see Table 3, Supplementary Figure S9) when normalized against 18S rRNA. However, following the application of conservative data filtering using the FDR–Benjamini–Hochberg algorithm, only HDAC5 (~1.25-fold) and Tgfbr1 (2.5-fold) survived as significant (but weak) decreases in expression (Table 3, and Supplementary Figure S9). This suggested that the changes in glucose tolerance, insulin signaling and lipogenesis were not indirect effects of changes in muscle mass and/or contractile function.

**DISCUSSION**

The biochemical and molecular characterization of RORs in cell culture and animal models has revealed that RORs play a critical role in the modulation of lipid homeostasis in a tissue-specific manner (1–5,31,32). Our previous studies reported that homozygous (male and female) staggerer mice (with decreased and dysfunctional expression of RORz in all organs) display reduced adiposity and are resistant to high-fat diet-induced obesity. The lean phenotype of staggerer mice was associated with significantly increased expression of genes involved in fatty acid oxidation (including PGC-1, lipin1, etc.), and significantly reduced expression of SREBP-1c (and several lipogenic genes) in all major metabolic tissues.

Surprisingly, studies investigating the role of RORz in the regulation of glucose homeostasis have not been reported. In this context, we were particularly interested in exploring the role of RORz1 in skeletal muscle, a major mass peripheral tissue that accounts for the majority of glucose disposal and lipid catabolism. We probed the role of RORz signaling in this major mass peripheral tissue, by the skeletal muscle-specific overexpression of truncated RORz1ΔDE (lacking the ligand-binding domain) to investigate the contribution of this peripheral tissue to the RORz phenotype. This construct has been described to operate in a dominant negative manner in several reports (21,22). This strategy also allows the probing of RORz-dependent gene expression in the absence of an identified native and/or synthetic ligand.
Figure 6. (A) A pictorial representation of the cross-talk between Akt2 and AMPK pathways, highlighting the implications for lipogenesis in skeletal muscle. (B) and (C) Western blot and densitometry analysis of AMPK and pAMPK in skeletal muscle of transgenic RORα1ΔDE and wt littermate control mice. (D) and (E) Western blot and densitometry analysis of AMPK and pAMPK in skeletal muscle of wt and staggerer (sg/sg) littermate control mice. (*P < 0.05, **P < 0.01).

Figure 7. (A) A pictorial representation of the cross-talk between the AMPK and fatty acid oxidation pathways, highlighting the implications for fatty acid oxidation in skeletal muscle. (B) Western blot analysis of ACC and pACC in skeletal muscle of transgenic and wt littermate control mice. Densitometry analysis of western blots (n = 3/group, mean ± SEM. **P < 0.01). (C) densitometry analysis of ACC and pACC in skeletal muscle of transgenic RORα1ΔDE and wt littermate control mice. (D) qPCR of PGC-1α and (E) CPT1b in skeletal muscle of transgenic and wt littermate control mice. Relative fold change is normalized against 18S mRNA (n = 6/group, mean ± SEM. *P < 0.05, **P < 0.01).
The human skeletal z-actin promoter was utilized to construct the muscle-specific vector. This promoter has been demonstrated to function in a cell/tissue-specific manner (10,11). As expected the transgene, RORz1ΔDE, was efficiently and specifically expressed in skeletal muscle of transgenic mice relative to other organ/tissues. Second, our RORz1ΔDE expression vector retains the native RORz1 amino-terminal AF-1 domain (i.e. AB region) and the native zinc finger region; these properties ensure that the NR targets the native RORz1 response elements in the skeletal muscle of transgenic mice. Giguere et al. (26) demonstrated that each ROR isoform displays isoform-specific DNA binding, and the amino-terminal domain and the zinc finger region work in parallel to confer sequence-specific DNA recognition and binding.

Expression profiling, coupled to analysis on the ingenuity platform identified a RORz1-regulated subset of genes that were involved in or associated with lipid metabolism, small molecule transport and biochemistry, cardiovascular and metabolic disease, carbohydrate metabolism, endocrine system disorders etc. This is in concordance with investigations in RORz-deficient mouse models and in vitro promoter/cell culture investigations that have demonstrated the involvement of RORz in a number of physiological process including atherosclerosis, lipid homeostasis, fat deposition, obesity, inflammation, immunity, etc (6). In the framework of this investigation, the primary signaling pathways regulated in skeletal muscle were glutathione metabolism (that tightly controls ROS levels and modulates insulin sensitivity), type 2 diabetic signalling and fatty acid biosynthesis. Notably, the LXR signaling (and RXR dependent) pathway was identified in the analysis, which controls lipogenesis and cholesterol homeostasis.

In concordance with the array analysis, qPCR analysis on the TLDA platform revealed significantly attenuated expression of the mRNAs (encoding the hierarchical regulators of lipogenesis), LXRz and SREBP-1c and several other downstream target genes involved in fatty acid biosynthesis in skeletal muscle. For example, we observed decreased expression of the downstream target genes, SCD1 and FAS. Moreover, in the context of lipogenesis, we observed the suppression of Acs14, Dgat2, Tip47, HIF-1 and Cd36. Acs14, the long chain acyl CoA synthetase, plays an important role in lipid metabolism by routing fatty acids to several different metabolic pools. Decreased Acs14 expression is associated with decreased triglycerides and fatty acids partitioning to di- and tri-acyl glycerol (33,34) and consistent with reduced Dgat2 (that catalyzes the final step in triglyceride production) and Tip47 (a perilipin family member that co-ordinates the storage of triacylglycerol (35). Furthermore, it has been reported that decreased Dgat2 expression is associated with reduced expression of SREBP-1c and SCD-1 and increased CPT-1 expression (see below), in concordance with our observations (36). Finally, suppression of HIF-1alpha is associated with reduced fatty acid uptake and biosynthesis, and in concordance with the suppression of the genetic program controlling lipogenesis and the decrease in the fatty acid translocase, CD36 (37).

In the context of the decrease in LXR and other NR signaling pathways, we completed TLDA analysis of the entire NR supergene family. This analysis identified decreases in the expression of the mRNAs encoding GR, PPARδ, TR2 and RORγ. These nuclear receptors have been implicated in the regulation of lipid homeostasis. We did not observe decreases in THR(α/β), FXR, VDR, etc. that were identified by ingenuity. We suggest that the detection of these pathways by ingenuity reflects secondary consequences of RORz-mediated dysregulation and the aberrant expression of NRs identified above.

As discussed, ingenuity analysis of the array data identified carbohydrate metabolism as a significantly regulated function. We observed the male tg-RORz1ΔDE mice display significantly increased fasting plasma glucose levels, impaired glucose tolerance and attenuated insulin-stimulated glucose uptake relative to wild-type littermates. Our qPCR TLDA profiling identified the significantly decreased expression of the mRNA encoding Akt2/PKB in skeletal muscle, after Bayes assignment of significance and stringent FDR filtering. Furthermore, the total amount of Akt and phospho-Akt protein was significantly reduced. In this context, several studies have demonstrated that, Akt2 plays a critical role in insulin-mediated glucose disposal in skeletal muscle. For example, Cho et al. (38) demonstrated that disruption of the Akt2 gene expression in mice was associated with insulin resistance and type2 diabetes-like syndrome. Subsequently, Garofalo et al. (39) showed mice lacking Akt2/PKB gene displayed a diabetic phenotype and age-dependant lipodystrophy.

Interestingly, the mild hyperglycemia (in the fasted state), glucose intolerance and impaired glucose uptake occur without any significant change in whole body insulin sensitivity (i.e. the insulin tolerance test) and/or change in GLUT1 and four expression. First, this does not appear to involve an insulin secretion defect as the transgene is not expressed in the pancreas and the mice develop hyperinsulinemia after a high-fat challenge (data not shown). It should be noted that muscle-specific deletion of an essential component (rictor) of the mTOR complex 2 leads to the attenuation of insulin-induced phosphorylation of Akt2 at Ser473. Moreover, these mice displayed glucose intolerance, however, insulin sensitivity remained unchanged (40). Similarly, our Tg-RORz1ΔDE mice displayed an attenuated insulin-mediated Ser473 phosphorylation of Akt and glucose uptake. In this context, Gonzalez and McGraw (41) demonstrated that ‘insulin signalling diverges into Akt-dependent and independent signals’. James and colleagues (42) recently stated that several studies underscore the pivotal role of Akt2 in GLUT4 function; however, many gaps remain in understanding the signaling cascades involved. For example, similar inconsistencies (between impaired glucose uptake and/or normal glucose tolerance and insulin sensitivity) have been described in other animal models that have perturbed expression and/or knockout in critical genes that regulate glucose homeostasis.
ChIP analysis revealed RORζ1 was directly involved in the regulation of Akt2. We identified several potential RORα1 response elements in the mouse Akt2 promoter between −2900 and −2600 nt upstream of the transcription start site. These sites were accommodated by the motif $R^A/T/A_R^A/R^A/T/A_R$ described by Giguere et al. (26), as an optimal RORζ1-binding site. ChIP identified functional RORζ1 recruitment to three sites (in close proximity) within this region. Further characterization of the Akt2 promoter is required to elucidate the mechanism mediating regulation by RORζ1.

Ingenuity identified AMPK signaling as a regulated pathway in the Tg mice overexpressing the dominant negative RORζ1. It has been well established that AMPK is master regulator of energy homeostasis through the suppression of ATP-consuming lipogenic pathways and by the enhancement of ATP producing catabolic pathways, including fatty acid oxidation in skeletal muscle tissue (43,44 and references therein). Moreover, Akt2/PKB is a potent modulator of AMPK activity. For example, Hahn-Windgassen et al. (28) showed that Akt/PKB regulates intracellular ATP levels through regulating AMPK activity. Furthermore, Planavila et al. (45) showed that mice treated with troglitazone (PPARγ agonist) in skeletal muscle were associated with increased Akt2/PKB and decreased AMPK activity. Moreover, recent investigations on Akt2/PKB and AMPK pathways in heart tissue showed that Akt2/PKB is a negative regulator of AMPK activity (27). Finally, the association between increased AMPK activity and the reduction of lipogenic gene expression and genes regulating fatty acid oxidation is well documented (46–49). Furthermore, the inhibitory effect of potent AMPK activators such as aminooimidazole carboxamide ribonucleotide (AICAR) or Metformin on lipogenesis in skeletal muscle has been described in both human and rodents (29,30). Consequently, in the context of our studies, we were particularly interested in examining the cross-talk between lipid homeostasis (lipogenesis and fatty acid oxidation), Akt2 signaling (and glucose tolerance) and AMPK signaling in a background of aberrant RORζ1 (NR) signaling.

Interestingly, the hyperglycemia and significantly decreased Akt2 (mRNA and protein) and phosphoAKT levels in the Tg-RORζ1ΔDE mice are associated with increased phosphoAMPK levels, in concordance with the studies discussed above. This is consistent with studies demonstrating type 2 diabetic patients also display normal AMPK signaling (50,51). Paradoxically, some studies report that increased AMPK activity mediates (only exercise induced) glucose transport. However, AMPK null mice exhibit typical exercise stimulated glucose uptake in skeletal muscle and AICAR does not induce glucose transport in muscle (52). Interestingly, we did not observe increases in pAMPK in the skeletal muscle of staggerer (sg/sg) mice that lack RORζ expression in all tissues (although we observed mild elevation in the levels of basal/total AMPK). Differential AMPK activity in these lines is not completely unexpected for several reasons. Firstly, we are comparing a line of mice overexpressing dominant negative RORα1 (in skeletal muscle), against a line of mice lacking RORζ1 expression. Second, in mice lacking NR expression, genes (and/or phenotypes) maybe silenced or derepressed depending on cofactor requirements, expression and/or promoter characteristics (53).

The increased AMPK activity in the Tg-RORζ1ΔDE mice did not only correlate with decreased Akt activity, but was also consistent with (i) attenuated mRNA expression of LXRα and SREBP-1c, and several other genes involved in fatty acid biosynthesis in skeletal muscle (44,54,55) and (ii) induction of the genes/pathways increasing fatty acid oxidation (56). For example, SCD1 deficiency has been reported to be associated with increased AMPK activity, fatty acid oxidation and reduced ceramide synthesis (41,42,57). In addition, in this interconnected regulatory milieu, we observed increased expression of two critical regulators of fatty acid oxidation, PGC-1α and CPT1b, concomitant with elevated levels of phospho-Ser79 ACC in the Tg-RORζ1ΔDE mice, relative to the wt littermate mice. This is also consistent with the observations (47–49,56 and references therein) that activated AMPK and AMPK agonists regulate PGC-1 and pACC expression.

Finally, overexpression of truncated RORζ in skeletal muscle resulted in very minor changes in the expression of genes regulating muscle mass, proliferation and differentiation. Interestingly, the analysis did identify HDAC5 as a differentially expressed gene. A recent study has demonstrated the link between AMPK, energy balance and transcriptional regulation of GLUT4 expression mediated by HDAC5. That study indicated that increased AMPK activity induced HDAC phosphorylation that reduces HDAC5 association with the GLUT4 promoter. Interestingly, in our mouse model, although insulin mediated phosphorylation of Akt2, and glucose uptake was perturbed; the association between increased AMPK activity and HDAC5 was maintained.

In conclusion our investigation reveals that the orphan nuclear receptor RORζ operates at the nexus of pathways controlling the association between lipid homeostasis (lipogenesis, and fatty acid oxidation), Akt2 signaling (glucose tolerance and uptake) and AMPK signaling.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

FUNDING
Research project grant from the National Health and Medical Research Council (NHMRC) of Australia, and the Diabetes Australia Research Trust (DART). GEOM is a Principal Research Fellow of the NHMRC, and Suryaparaksh Raichur was a recipient of an International Postgraduate Research Scholarship (IPRS).

Conflict of interest statement. None declared.
REFERENCES


