AP2-NR4A3 transgenic mice display reduced serum epinephrine due to increased catecholamine catabolism in adipose tissue

R. Grace Walton1, Xiaolin Zhu1, Ling Tian1, Elizabeth B. Heywood2, Jian Liu1, Helliner S. Hill1, Jiarong Liu3, Dennis Bruemmer2, Qinglin Yang1, Yuchang Fu1, and W. Timothy Garvey1,4

1Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama, USA
2Saha Cardiovascular Research Center and Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, Kentucky, USA
3Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama, USA
4Birmingham Veterans Affairs Medical Center, Birmingham, Alabama, USA

*These authors contributed equally.

Keywords
NR4A3 transgenic mice, NOR-1, MINOR, Type 2 Diabetes, lipolysis, catecholamine catabolism, epinephrine, monoamine oxidase

Correspondence
R.Grace Walton, Ph.D.
College of Health Sciences
University of Kentucky
900 S. Limestone, CTW 433
Lexington, KY 40536
Tel: 859-218-0880
E-mail: r.grace.walton@uky.edu

Running foot: Decreased epinephrine in AP2-NR4A3 mice
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdipoQ</td>
<td>Rodent adiponectin</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>AdQ-R1</td>
<td>Adiponectin receptor 1</td>
</tr>
<tr>
<td>AdQ-R2</td>
<td>Adiponectin receptor 2</td>
</tr>
<tr>
<td>AP2</td>
<td>Adipocyte fatty acid binding protein, FABP4</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CEPB-α</td>
<td>CCAAT/enhancer-binding protein-α</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual emission x-ray absorptiometry</td>
</tr>
<tr>
<td>FABP4</td>
<td>Adipocyte fatty acid binding protein, AP2</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Insulin receptor substrate-2</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MAO-A</td>
<td>Monoamine oxidase-A</td>
</tr>
<tr>
<td>MAO-B</td>
<td>Monoamine oxidase-B</td>
</tr>
<tr>
<td>NBRE</td>
<td>NGFI-B response element</td>
</tr>
<tr>
<td>NET</td>
<td>Norepinephrine transporter</td>
</tr>
<tr>
<td>NR4A1</td>
<td>Nuclear receptor 4A1, Nur77</td>
</tr>
<tr>
<td>NR4A2</td>
<td>Nuclear receptor 4A2, Nurr1</td>
</tr>
<tr>
<td>NR4A3</td>
<td>Nuclear receptor 4A3, NOR-1, MINOR</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PPAR-γ coactivator-1α</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element-binding protein-1c</td>
</tr>
<tr>
<td>SSAO</td>
<td>Semicarbazide-sensitive amine oxidase</td>
</tr>
</tbody>
</table>
The NR4A orphan nuclear receptors function as early response genes to numerous stimuli. Our laboratory has previously demonstrated that over-expression of NR4A3 (NOR-1, MINOR) in 3T3-L1 adipocytes enhances insulin-stimulated glucose uptake. To assess the \textit{in vivo} effect of NR4A3 on adipocytes, we generated transgenic mice with NR4A3 over-expression driven by the adipocyte AP2 promoter (AP2-NR4A3 mice). We hypothesized that AP2-NR4A3 mice would display enhanced glucose tolerance and insulin sensitivity. However, AP2-NR4A3 mice exhibit metabolic impairment, including increased fasting glucose and insulin, impaired glucose tolerance, insulin resistance, decreased serum free fatty acids, and increased LDL-cholesterol. Furthermore, AP2-NR4A3 mice display a significant reduction in serum epinephrine due to increased expression of catecholamine catabolizing enzymes in adipose tissue, including monoamine oxidase-A. Furthermore, enhanced expression of monoamine oxidase-A is due to direct transcriptional activation by NR4A3. Finally, AP2-NR4A3 mice display cardiac and behavioral alterations consistent with chronically low circulating epinephrine levels. In conclusion, overexpression of NR4A3 in adipocytes produces a complex phenotype characterized by impaired glucose metabolism and low serum catecholamines, due to enhanced degradation by adipose tissue.

\section*{INTRODUCTION}
The orphan receptor NR4A subgroup of the nuclear hormone receptor superfamily is comprised of 3 genes, NR4A1 (or Nur77), NR4A2 (or Nurr1), and NR4A3 (NOR-1 or MINOR). NR4A family members are early response genes whose expression is induced in a cell-type specific manner by numerous stimuli, including: fasting, exercise, inflammation, hypothalamic-pituitary-adrenal-axis (HPA-axis) hormones, tyrosine-derived neurotransmitters, and cAMP analogs. NR4A receptors modulate expression of steroidogenic, gluconeogenic, glycolytic, and β-oxidative genes in both the HPA-axis and target tissues (24, 28).

While NR4A family members are expressed in numerous tissues, NR4A3 expression is more limited with high levels detected in metabolically active tissues such as muscle and adipose tissue. We have shown that NR4A3 is depleted in muscle and fat from several insulin-resistant rodent models (11); however, it is up-regulated by insulin in human vastus lateralis muscle and expression in muscle is increased in insulin-sensitive versus insulin-resistant subjects (41). In addition, we have demonstrated that NR4A3 enhances insulin-stimulated glucose transport and insulin signaling when overexpressed \textit{in vitro} in 3T3-L1 adipocytes (11) and C2C12 muscle cells (42). These data indicated that NR4A3 might enhance insulin sensitivity, raising the possibility that NR4A3 agonism could constitute a viable pharmacological target for insulin-sensitizing drugs, analogous to thiazolidinedione agonism of PPAR-γ nuclear receptors.

Investigators have generated mouse models with global overexpression or knockout of NR4A3, although these models have not been carefully assessed for metabolic phenotypes. One line of NR4A3 null mice exhibited abnormal...
hippocampal development, increased predisposition to excitotoxic glutamate receptor kainic acid-induced seizure, inner ear defects, and aberrant circling behavior (31, 32). A second NR4A3 knockout was embryonic lethal in homozygous mice due to incomplete gastrulation while heterozygous mice appeared to be normal (8). Transgenic global over-expression of NR4A3 produced a marked reduction in body weight (~50%), atrophy of the spleen and thymus, and a live birth rate of less than 50% (15).

The in vivo physiological effects of adipose NR4A3 over-expression in mice are unknown. Based on our results in 3T3-L1 adipocytes (11), we hypothesized that adipose NR4A3 over-expression would enhance insulin sensitivity in adipose tissue and lead to systemic improvements in glucose tolerance. To test this hypothesis, we have generated NR4A3 transgenic mice using the AP2 promoter to drive expression in adipocytes. Surprisingly, we observed that these animals have metabolic and other impairments that are likely due to pronounced reductions in circulating catecholamines.

METHODS

Generation of transgenic animals. The entire human NR4A3 gene coding sequence was cloned by Dr. Lihong Luo, affixed with a human growth hormone tail (kindly provided by Dr. Yuqing Eugene Chen, University of Michigan Medical Center, Ann Arbor MI) and then inserted into the AP2 promoter DNA construct (5.4 kb promoter/enhancer, with a V5 viral epitope tag) (kindly provided by Dr. Bruce Spiegelman, Harvard Medical School, Boston MA) with Hind III and Not I restriction
enzyme sites. DNA injection and embryo transfer were performed by the UAB Transgenic Animal/Embryonic Stem Cell Core, Dr. Robert Kesterson, director. Mice were generated on a C57BL/6 (Taconic Farms) background.

Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Unless otherwise noted, all experiments were performed on male animals. Experiments employed multiple animal cohorts. In order to produce transgenic animals and wild-type littermate controls, breeding pairs consisted of one heterozygous AP2-NR4A3 transgenic animal and one pure C57BL/6 (Taconic Farms). All animals were maintained under standard conditions (22±2°C, 12-hour light cycle) and given ad libitum access to water and either standard rodent diet (Harlan Teklad 7913 Irradiated Modified 6% Mouse/Rat Sterilizable Diet) or high fat diet (Research Diets D-12492, 60% kcal from fat, 20% kcal from protein, 20% kcal from carbohydrate). Body weight and food intake were measured weekly beginning at age 8 weeks and ending at age 30 weeks. Animals were euthanized by decapitation without anesthesia. Male animals were sacrificed at 33-35 weeks of age. Tissues and whole trunk blood were harvested and gonadal fat pads were weighed. All tissues except trunk blood were quickly placed into liquid nitrogen and stored at -80°C until needed. Whole trunk blood was centrifuged for 30 minutes at 5000 x g, 4°C, and serum was collected. All rtPCR, real-time rtPCR, western blotting, chromatin immunoprecipitation, and primary adipocyte culture experiments were performed using gonadal fat.
PCR. Genotyping, quantitative real-time PCR, and reverse transcriptase PCR were performed with the following primers: forward 5'-GGA TCC AAA CTC ATT ACT AAC CGG TA-3' and reverse 5'-ATA TCC AAG CCT TAG CCT GCC TGT-3', with forward primer complimentary to the transgene V5 tag and reverse primer complimentary to the human NR4A3 gene. Tail snips, followed by phenol:chloroform genomic DNA extraction, were used for genotyping via traditional PCR and assessment of gene copy number via quantitative real-time PCR. For reverse transcriptase PCR, RNA was extracted from gonadal fat using TRIzol reagent (Invitrogen) according to manufacturer’s protocol. Samples were treated with DNAse I (amp grade, Invitrogen), and then used as template for the production of cDNA (SuperScript III, Invitrogen). PCR was then performed on cDNA and amplification product resolved on 1.5% agarose gel. For real-time rt-PCR, adipose, adrenal, and other tissue RNA was extracted using Qiagen RNEasy Lipid Tissue Mini Kit (Qiagen USA, Valencia, CA), reverse transcription was performed using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and double-stranded DNA amplicon was detected using SYBR GreenER reagent (Invitrogen, Carlsbad, CA). Real-time RT-PCR data were normalized to 18-S RNA using the 2^ΔΔCT method.

Chromatin immunoprecipitation. Gonadal fat from wild-type and transgenic mice was pooled (n=2-3 per genotype) in order to increase chromatin yield. Adipose tissue was homogenized, cross-linked with 37% formaldehyde, cells were lysed in 50 µl lysis buffer plus protease inhibitor cocktail, and then sonicated using 24 cycles of 30 seconds on and 30 seconds off. For Chromatin immunoprecipitation, 50 µg of
chromatin was used. AP2-NR4A3 chromatin was used for precipitation with mouse IgG as negative control. Samples were immunoprecipitated with 5 µg NR4A3 antibody (R&D Systems, PP-H7833), reverse crosslinked, and DNA was purified and eluted in 150ul DNA elution buffer according to MAGnify kit instructions (Invitrogen Life Technologies, Carlsbad, CA). DNA was stored at -20C until ready to assay for NR4A3 binding to the MAO-A promoter. PCR results are expressed as amplification relative to input control, in which DNA is obtained from chromatin that has been reverse-crosslinked, but not immunoprecipitated. PCR primers flank a true nerve growth factor IB response element (NBRE) located -3458 to -3268 upstream of the monoamine oxidase-A transcription start site (forward 5’-CCT AGG GCC TTG AAA AG-3’, reverse 5’-TCC AGC ACC AGA AGC AGA G-3’).

**Western blotting.** Gonadal fat protein was extracted with Sigma CelLytic Mammalian Tissue Lysis Reagent and separated by SDS-polyacrylamide gel electrophoresis. For NR4A3, protein was transferred onto nitrocellulose membranes and incubated overnight at 4°C with 5% non-fat milk in TBS. Membranes were then incubated with NR4A3 antibody (1:500; R&D Systems, PP-H7833) for 1 hour followed by incubation with horseradish peroxidase secondary antibody for 1 hour. Membranes were washed with TBS (with 0.1% Tween 20) and protein was detected by chemiluminescence (Enhance, NEN Life Science) and quantified by densitometry. For monoamine oxidase-A (MAO-A), protein was transferred onto nitrocellulose membranes and incubated for 1 hour at room temperature with Odyssey blocking buffer (Li-Cor, Lincoln, NE). Membranes were then incubated with MAO-A antibody (1:200; Santa Cruz Biotechnology, MAO-A(T-19); sc-18397) for 1
hour followed by incubation with IRDye 800CW Donkey anti-Goat IgG (Li-cor 925-32214) for 30 minutes. Membranes were washed with TBS (with 0.1% Tween 20) and protein was detected by near-infrared fluorescence (Odyssey, Li-cor) and quantified by densitometry.

**Glucose tolerance test (GTT).** Seventeen week old and 31 week old animals were fasted overnight with free access to water. At 9:00 a.m., animals were weighed and given an intra-peritoneal injection of D-(+)-glucose (100g glucose/L; 10μl/g body weight). Blood glucose was measured at baseline (approximately 1 minute prior to injection), and at 30, 60, 90, and 180 minutes post-injection (HemoCue glucose 201 glucometer, HemoCue USA).

**Insulin tolerance test (ITT).** Eighteen and 32 week old animals were fasted for 4 hours and then weighed. Animals were then given an intra-peritoneal injection of 1.5U (male mice) of rapid acting insulin/kg body weight (Humalog® lispro; Eli Lilly & Co.). Blood glucose was measured at baseline (approximately 1 minute prior to injection), and at 30, 60, 90, and 180 minutes post-injection. Animals were not given access to food or water during GTT and ITT.

**Dual-energy X-ray absorptiometry (DXA).** Mice were anesthetized with 3% isoflurane and body composition was analyzed using the GE Lunar Piximus (Madison, WI), software version 1.4, in the Small Animal Physiology core laboratory of the UAB Diabetes Research Center, directed by Dr. Timothy R. Nagy, according to previously published protocols (25). Animals were scanned at age 8, 16, and 30 weeks (males fed standard diet); or age 16 weeks (males fed high fat diet).
**In vivo lipolysis and anti-lipolysis.** The lipolytic effect of β-adrenergic agonism was assessed by intra-peritoneal delivery of isoproterenol (1mg/kg body weight, 10 mg/kg body weight, or 15 mg/kg body weight), decapitation exactly 15 or 30 minutes later, and determination of serum free fatty acids. In order to assess insulin’s ability to suppress adipocyte lipolysis, male animals (age 33-35 weeks, maintained on standard rodent diet) were fasted for 4 hours, weighed, and injected with 1.5U Humalog lispro insulin/kg body weight. Mice were sacrificed exactly 60 minutes after insulin injection, and whole trunk blood was collected for analysis. Analytes included insulin, free fatty acids, epinephrine, and norepinephrine (methods below).

**Behavior.** Behavioral assays were performed in the UAB Neuroscience Behavioral Assessment Core. Feeding behavior was monitored on the LABORAS automated animal behavior recognition system (Metris, The Netherlands). Open field testing was performed on a 42 cm X 42 cm platform, with Noldus Ethovision (3.1) (Noldus, The Netherlands) tracking software.

**Energy expenditure and body temperature.** Experiments were performed in the UAB DRC Animal Physiology Core. Total energy expenditure was analyzed using indirect calorimetry (LabMaster; TSE Systems) over 2 days, according to previously published protocols (21). Animals were allowed to acclimate in metabolic cages for 48 hours prior to measurement. Twenty-four hour body temperature was measured using Mini-mitter telemetry (ER-4000 Respironics, Bend, OR). Mini-mitters were surgically implanted in the peritoneum 2 weeks prior to telemetry, and animals were allowed to acclimate in cages for 2 days prior to measurement. Data
were processed with LabVIEW 5.0 (National Instruments, Austin, TX). Rectal body temperatures were measured with an Oakton Acorn Temp J-K-T Thermocouple Thermometer (Cole-Parmer USA, Vernon Hills, IL) equipped with a small, flexible round-tipped probe.

Pulse, blood pressure, and cardiac function. Tail-cuff pulse and blood pressure were measured in un-anesthetized, awake mice using the Hatteras MC-4000 Blood Pressure Analysis System (Hatteras Instruments, Cary, NC). Reported data represent means of approximately 45 observations per mouse. Echocardiographic measurement was performed with the high resolution echocardiography analysis system for small animals (Vevo 770™, VisualSonics, Canada). Mice were anesthetized with 2% isoflurane inhalation in O₂. Two-dimensional short-axis view and M-mode tracings of the left ventricle were obtained with a 30 MHz transducer. Echocardiography was analyzed with the Advanced Cardiovascular Analysis Package from the manufacturer of the VEVO 770™ system (VisualSonics), based on previously published guidelines (19).

Serum assays. Serum insulin, leptin, and adiponectin were measured by double-antibody RIA (Linco sensitive rat insulin RIA, Millipore mouse leptin RIA, Millipore mouse adiponectin RIA). Serum corticosterone and fecal corticosterone metabolites were measured using MP Biomedicals rat/mouse corticosterone RIA. Fecal corticosterone was extracted by homogenization in 80% methanol, centrifugation, and removal of supernatant. Free fatty acids, total cholesterol, and triglycerides were assessed by in vitro enzymatic colorimetry (HR Series NEFA-HR(2), Cholesterol-E, and L-Type TG H kits from Wako Diagnostics).
Serum catecholamines were measured in the CMN/KC Neurochemistry Core Lab, Vanderbilt University, Nashville, TN. First, serum catecholamines are adsorbed onto solid Al₂O₃, and then desorbed from the Al₂O₃ using 200 µl 0.1 N acetic acid. Biogenic amines are determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.5) electrochemical detector. Twenty µl samples were injected using a Water 717+ autosampler onto a Phenomenex Nucleosil (5 µm SA 100 Å) C18 HPLC column (150 x 4.60 mm).

Apolipoprotein assays were performed on four pooled serum samples from 16 transgenic mice and four pooled samples from 16 wild-type mice. Pooled samples were analyzed using the lipoprotein autoprofiler method (6). Density gradient ultracentrifugation was used to separate the major lipoprotein fractions in the serum. Effluent was continuously removed from the bottom of the density gradient and analyzed using enzymatic colorimetric cholesterol and phospholipid kits from Wako Diagnostics.

*Primary adipocyte culture.* Primary adipocyte culture was performed as previously described (12, 38). Gonadal fat was removed and cells were isolated by collagenase digest in bicarbonate buffered DMEM with 4% BSA and 5 mM glucose, then re-suspended to a final 5% (vol/vol) cell concentration. For measurement of insulin-stimulated 2-deoxyglucose transport (2-DOG), cells were incubated with or without insulin for 60 minutes, pulsed with 3H-2-deoxyglucose for 3 minutes, and then centrifuged at 14,000 x g for 30 seconds. 2-DOG radioactivity was measured in adipocyte pellets. Calculation of intracellular 2-DOG was corrected for non-specific carryover and glucose uptake due to simple diffusion using radiolabeled L-glucose.
In order to correct for cell surface area, cell diameters were measured via microscopy. For *in vitro* lipolysis and anti-lipolysis, a 5% (vol/vol) adipocyte suspension was prepared, aliquots were treated with adenosine deaminase for 5 min, and then treated with either 10 µM isoproterenol, 0.5 nM insulin, both insulin and isoproterenol, or buffer only. The cells were incubated at 37°C for 1 hour, then centrifuged at 3000 x g for 10 minutes and the infranatant was collected and used for free fatty acids measurement (Wako HR Series NEFA-HR(2)). In order to determine whether mouse adipose tissue is capable of measurable epinephrine catabolism, 0.2 g of mouse gonadal and inguinal fat was excised, minced, and placed in 2 ml of sterile Dulbecco’s medium (DMEM) containing 20 mM HEPES, pH 7.4, 5mM glucose, 1% (w/v) bovine serum albumin (BSA), and 12.5, 25, or 100 pg/µl epinephrine (Epi-pen, Dey Pharma). Tissue was incubated at 37°C with gentle rotation and 100 µl of culture media was collected after 8, 15, 30, and 60 minutes. Media epinephrine was measured by HPLC, as described above. It was determined that the greatest differences were observed using 25 pg/µl epinephrine for 15 minutes.

**Immunohistochemistry (IHC).** IHC was performed in the UAB Neuroscience Molecular Detection Core. Adrenal glands and surrounding fat were fixed in 4% paraformaldehyde, dehydrated with ethanol, paraffin embedded, sliced (7 µm), and mounted on slides. Purified Mouse Monoclonal NR4A3 Antibody (Abgent, San Diego, CA) was used as primary antibody. Biotinylated Donkey anti-mouse
secondary antibody (Jackson ImmunoResearch, West Grove, PA) was applied, and detection was performed with 3,3-Diaminobenzidine.

Statistics. All experimental cohorts consisted of same-sex, same-age transgenic animals with wild-type littermate controls. Unless otherwise indicated, all statistical analyses represent comparisons between transgenic and wild-type mice. Unless otherwise noted, data are reported as mean ± standard error of the mean. Mice were not matched by body weight or lean mass. Thus, ANCOVA was used to estimate the contribution of genotype versus body weight in energy expenditure analysis. Analysis of GTT, ITT, and body weight employed the following tests: repeated measures ANOVA (RMANOVA), student’s t-test of area under the curve, and student’s t-test of change from baseline. Food intake was analyzed using Wilcoxon rank sum because food intake/lean mass was not normally distributed. All other experiments were analyzed using student’s t-test. Statistical tests were considered significant at $P < 0.05$. Statistical outliers greater than 2 standard deviations from the mean were removed. Appropriate statistical software, including JMP (SASS Institute) was used for all analyses.

RESULTS

Verification of transgene insertion and function. We utilized a transgene consisting of the human NR4A3 gene driven by the AP2 promoter, which is highly expressed in adipose tissue (Fig. 1A). Transgenic mice were genotyped for the NR4A3 transgene by PCR, using a 3’ primer annealing to a V5 coding region. Transcriptional activity of the NR4A3 transgene in gonadal fat was verified by
reverse transcriptase PCR (Fig. 1B). NR4A3 protein levels in gonadal fat were
significantly increased compared to those observed in wild-type mice (P < 0.05)
(Fig. 1C and D). Quantitative PCR results indicated numerous transgene copies
(data not shown).

Many of the phenotypic differences between AP2-NR4A3 and wild-type mice
became more pronounced as the mice aged. Therefore, we measured metabolic
parameters (body weight, body composition, glucose and insulin tolerance) at ages
16 and 30 weeks. All other measures were performed at age 30 to 33 weeks.

When fed standard rodent diet, AP2-NR4A3 transgenic mice weigh more
than wild-type littermates, with no differences in percent fat, percent lean, or body
temperature. AP2-NR4A3 males maintained on standard diet weighed significantly
more than their wild-type littermates from age 8 to 16 weeks (P < 0.05 for genotype,
P < 0.001 for time, time x genotype NS, post-hoc for genotype P < 0.05 at age 10,
11, and 12 weeks, RMANOVA), with this pattern persisting from age 20 to 30 weeks
(P < 0.01 for genotype, P < 0.001 for time, time x genotype NS, post-hoc tests NS,
RMANOVA, post-hoc P < 0.05 for genotype at 26, 27, 28, 29, and 30 weeks old)
(Fig. 2A). Absolute weekly food intake values tended to be slightly higher in
transgenic males from 9 to 16 weeks of age (P = 0.1, NS) and from 20 to 29 weeks
of age (P = 0.09, NS) (data not shown). However, average weekly food intake per
gram body weight tended to be lower in transgenic animals from 9 to 16 weeks of
age (P = 0.07, NS, Wilcoxon rank sum, data not shown) and was significantly lower
from 20 to 29 weeks of age (P < 0.01, Wilcoxon rank sum, data not shown).

Because mice underwent stressful glucose- and insulin-tolerance tests at age 17
and 18 weeks, body weight and food intake were not measured during this time frame.

At 8 weeks of age, body composition data indicated that male AP2-NR4A3 mice fed standard diet tended to weigh more than wild-type mice, with a trend toward greater mean lean mass ($P = 0.054$). However, no differences in fat mass or percent fat were observed at 8 weeks of age. At 16 weeks of age, transgenic mice displayed a trend toward higher lean mass ($P = 0.05$), with no difference in fat mass or percent fat. At 30 weeks of age, AP2-NR4A3 mice displayed significantly greater lean mass ($P < 0.05$), a trend toward greater fat mass ($P = 0.1$, NS), and no difference in percent fat. Thus, AP2-NR4A3 weigh more than wild-type littermates, with significantly increased lean mass and a non-significant trend toward increased fat mass driving this difference. However, AP2-NR4A3 were not leaner than wild-type littermates, as there was no difference in percent fat at age 8, 16, or 30 weeks. When animals were sacrificed (between 33 and 35 weeks of age), AP2-NR4A3 mice tended to have larger gonadal fat pads, but this difference was not statistically significant. Body composition data are shown in Table 1.

Table 1. Body composition in AP2-NR4A3 mice at 16 and 30 weeks of age. Mice were fed standard rodent diet.

<table>
<thead>
<tr>
<th>Age 16 weeks</th>
<th>AP2-NR4A3 (N=19)</th>
<th>Wild-type (N=19)</th>
<th>$P =$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>$34.2 \pm 0.87$</td>
<td>$32 \pm 0.85$</td>
<td>$0.08$</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>$23.1 \pm 0.34$</td>
<td>$22.1 \pm 0.34$</td>
<td>$0.054$</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>$8.2 \pm 0.53$</td>
<td>$7.2 \pm 0.50$</td>
<td>$0.18$</td>
</tr>
<tr>
<td>% Fat</td>
<td>mean, median</td>
<td>25.7, 25.7</td>
<td>23.9, 25.1</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Age 30 weeks</td>
<td>AP2-NR4A3 (N=16)</td>
<td>Wild-type (N=17)</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>42.8 ± 1.2</td>
<td>39.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>25.6 ± 0.48</td>
<td>23.2 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>14.3 ± 0.73</td>
<td>12.5 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>% Fat</td>
<td>mean, median</td>
<td>36.5, 37.6</td>
<td>34.4, 36.3</td>
</tr>
</tbody>
</table>

AP2-NR4A3 transgenic mice displayed no significant differences in rectal temperature at 9:00 a.m. or 8:00 p.m., nor were there any differences in core body temperature as assessed by mini-mitter telemetry. Similarly, following placement in 4°C for 4 hours, AP2-NR4A3 rectal temperature was the same as wild-type (body temperature data not shown).

When fed standard rodent diet, AP2-NR4A3 transgenic mice display poor glucose tolerance and insulin resistance. AP2-NR4A3 transgenic mice maintained on a standard diet had elevated fasting glucose compared to wild-type littermates at age 17 weeks ($P < 0.05$), and exhibited impaired glucose tolerance. GTT area under the curve (AUC) blood glucose was significantly greater in transgenic animals compared to wild-type ($P < 0.05$). Accordingly, RMANOVA analysis indicated significantly different glucose responses between groups ($P < 0.01$). Insulin tolerance did not differ between groups at age 18 weeks when assessed by either AUC or RMANOVA (17 and 18 week data not shown).
At 31 weeks of age, transgenic animals continued to display increased fasting glucose compared to wild-type littermates (mean value for transgenic animals=162.3 ± 8.0 mg/dL, mean value for wild-type animals=140.7 ± 7.0 mg/dL, P < 0.05) (Fig. 2B). Furthermore, transgenic animals remained glucose intolerant compared to wild-type (genotype P < 0.01, RMANOVA), with a significant interaction between time and genotype (P < 0.01, RMANOVA), and a significant effect of genotype at times 60 and 90 minutes (post-hoc) (Fig. 2C). At 33-35 weeks of age, fasting insulin was also elevated in AP2-NR4A3 animals compared to wild-type (mean value for transgenic animals=0.93 ± 0.09 ng/ml, mean value for wild-type animals=0.65 ± 0.08 mg/dL, P < 0.05) (Fig. 2D). Versus control mice, transgenic mice were also insulin resistant at age 32 weeks as assessed by insulin tolerance test (RMANOVA: genotype P < 0.05, time P < 0.001, time x genotype P < 0.05, post-hoc tests for effect of genotype P < 0.05 at 30, 60 and 90 minutes). Accordingly, glucose clearance AUC during the ITT was significantly smaller in AP2-NR4A3 mice (mean AUC value for transgenic animals=-237.5 ± 34.2, mean AUC value for wild-type animals=-388.33 ± 59.3, P < 0.05) (Fig. 2E and 2F).

Male AP2-NR4A3 and wild-type mice were also fed a high fat diet. The transgenic mice continued to display increased body weight from age 9-16 weeks (P < 0.05) with no differences in percent fat when compared with wild-type (data not shown). Differences in body weight, fasting glucose, glucose tolerance, and insulin tolerance between wild-type and transgenic mice were minimized with longer term (>16 weeks) high fat feeding in comparison to results obtained with normal chow, and were not statistically significant. Because we observed no differences in fasting
glucose, GTT, or ITT between AP2-NR4A3 and wild type mice, we did not proceed to measure serum catecholamines or perform lipid profiles in high fat fed mice.

When fed standard rodent diet, AP2-NR4A3 transgenic mice are dyslipidemic. We observed significant differences in fasting serum lipids at age 33 to 35 weeks (Table 2). Free fatty acids were decreased in transgenic mice versus wild-type ($P < 0.05$), with no difference in triglycerides. Paradoxically, total cholesterol was significantly higher in transgenic mice ($P < 0.001$), with increased LDL-cholesterol accounting for the difference in total cholesterol ($P < 0.001$). VLDL-cholesterol and HDL-cholesterol did not differ between groups. When mice were maintained on a high fat diet, fasting serum lipids did not differ between groups.

Table 2. Serum Lipids in AP2-NR4A3 mice at age 33-35 weeks.

<table>
<thead>
<tr>
<th>Lipid analyte</th>
<th>AP2-NR4A3</th>
<th>Wild-type</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>1.15 ± 0.04</td>
<td>1.32 ± 0.06</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>105.9 ± 6.7</td>
<td>118.4 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>117.9 ± 4.6</td>
<td>96.0 ± 3.9</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>73.7 ± 6.1</td>
<td>69.6 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>35.1 ± 1.2</td>
<td>22.0 ± 0.94</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>VLDL-cholesterol (mg/dL)</td>
<td>6.5 ± 0.41</td>
<td>5.4 ± 0.53</td>
<td>NS</td>
</tr>
</tbody>
</table>

For free fatty acids, triglycerides & total cholesterol N = 15 AP2-NR4A3, 15 wild-type. For HDL, VDL, and VLDL N= 4 pooled serum samples from each group.

AP2-NR4A3 transgenic mice have normal serum leptin and adiponectin, and normal in vitro insulin-stimulated glucose transport and lipolysis/anti-lipolysis. Because AP2-NR4A3 mice displayed dyslipidemia and decreased glucose and
insulin tolerance when maintained on standard rodent diet, we assessed adipose
tissue products that modulate differentiation, glucose uptake, lipid transport,
lipolysis and inflammation. Serum leptin and adiponectin did not differ between
AP2-NR4A3 and wild-type mice. We performed real-time rtPCR on gonadal fat for
the following mRNA transcripts: PPARγ, PGC-1α, SREBP-1c, CEBPα, CD36,
LDLR, AP2/FABP4, ABCA1, perilipin, HSL, IR, IRS-1, IRS-2, GLUT-4, AdipoQ,
AdQ-R1, AdQ-R2, leptin, and NR4A2 (Nurr1), and observed no significant
differences. However trends were observed for the following gene transcripts: IL-6
(transgenic was higher, $P = 0.06$, NS) and NR4A1 (Nur77) (transgenic was higher,
$P = 0.1$, NS). Furthermore, gonadal adipocyte diameter did not differ between
transgenic and wild-type mice (data not shown). We also employed primary gonadal
adipocyte culture in order to assess insulin-stimulated glucose transport and
lipolysis/anti-lipolysis and observed no differences between transgenic and wild-
type.

AP2-NR4A3 transgenic mice do not differ from wild-type during in vivo anti-
lipolysis and lipolysis despite reduced serum catecholamines. To assess the anti-
lipolytic effect of insulin in vivo, animals were injected with insulin and blood was
obtained 1 hour later for measurement of free fatty acids. Since endogenous
catecholamines induce lipolysis, serum norepinephrine and epinephrine were
measured as covariates. Surprisingly, AP2-NR4A3 mice exhibit a striking reduction
in serum epinephrine following insulin delivery. Mean serum norepinephrine was
35.7 ± 2.1 pg/μl in AP2-NR4A3 versus 43.9 ± 2.8 pg/μl in wild-type ($P < 0.05$; Fig.
3A), while mean serum epinephrine was 5.1 ± 0.5 pg/μl in AP2-NR4A3 versus 10.3
± 1.1 pg/μl in wild-type (P < 0.001; Fig. 3B). Post-injection insulin levels did not differ between groups (Fig. 3C). Furthermore, following insulin delivery, serum free fatty acids were similar in transgenic and wild-type mice (Fig. 3D), despite significantly decreased serum catecholamines in transgenic animals. Finally, we performed in vivo lipolysis experiments in order to assess the whole-animal lipolytic response to isoproterenol; in numerous experiments using a range of doses and time points, AP2-NR4A3 tended to display increased free fatty acids in response to β-adrenergic agonism, but the differences were not statistically significant (Fig. 3D).

**AP2-NR4A3 transgenic mice have highly reduced serum epinephrine due to up-regulation of catecholamine catabolism in adipose tissue.** Since we observed that serum catecholamines were decreased post-insulin injection during in vivo anti-lipolysis experiments, we assessed whether untreated animals would display differences in serum epinephrine. Indeed, AP2-NR4A3 mice were found to have dramatically reduced serum epinephrine under basal conditions (AP2-NR4A3 mean 3.64 ± 0.36 pg/μl, wild-type mean 5.65 ± 0.33 pg/μl, P < 0.001) (Fig. 4A). However, norepinephrine did not differ between transgenic and wild-type mice under basal conditions (AP2-NR4A3 mean 8.94 ± 1.1 pg/μl, wild-type mean 9.18 ± 0.92 pg/μl, NS) (data not shown).

Adipose tissue has been shown to participate in catecholamine catabolism and subsequent clearance (30, 35). Thus, we first determined whether cultured adipose tissue slices from wild-type mice could clear measurable quantities of epinephrine from culture media. Indeed, epinephrine is rapidly cleared from adipose tissue culture media (≥15 pg/μl in 15 minutes, data not shown). We next sought to
determine whether epinephrine catabolism is increased in cultured AP2-NR4A3 adipose tissue versus wild-type. Indeed, following a 15 minute incubation (25 ng epinephrine, 0.1 g adipose tissue per ml culture media), transgenic adipose tissue degraded 139.5 ng epinephrine/g tissue while wild-type adipose tissue degraded 112.0 ng epinephrine/g tissue \((P < 0.05)\) (Fig. 4B).

We proceeded to determine adipose tissue gene expression of catecholamine catabolism enzymes using real-time rtPCR (see Fig. 5 for overview of catecholamine catabolism). In AP2-NR4A3 mice, monoamine oxidase-A (MAO-A) gene expression was highly up-regulated compared to wild-type \((P < 0.001)\).

Similarly, gene expression of monoamine oxidase-B (MAO-B), catechol-O-methyltransferase (COMT), and renalase were significantly up-regulated in AP2-NR4A3 mice \((P < 0.01\) for each gene) (Fig. 4C). There were no observed differences in semicarbazide sensitive amine oxidase (SSAO) expression.

In keeping with increased expression of amine oxidases and COMT, AP2-NR4A3 adipose tissue also exhibited a trend toward increased expression of the catecholamine transporters, extraneuronal norepinephrine transporter (NET; \(P = 0.08, NS\)) and organic cation transporter-1 (OCT-1; \(P = 0.06, NS\)). Finally, we observed alterations in adrenergic receptor gene expression, with AP2-NR4A3 mice having reduced \(\beta\)-3 adrenergic receptor \((P < 0.05)\) and increased \(\alpha\)-1 adrenergic receptor expression \((P < 0.01)\) (Fig. 4C).

Because MAO-A gene expression was highly increased in AP2-NR4A3 adipose tissue, we proceeded to measure MAO-A protein levels by western blot. There was a trend toward increased MAO-A protein in AP2-NR4A3 transgenic
adipose tissue (AP2-NR4A3 8.36 AU versus wild-type 5.48 AU, \( P = 0.1, \text{NS} \)) (Fig. 4D and E). We next determined whether NR4A3 directly interacts with the MAO-A promoter. Indeed, the MAO-A promoter contains an NR4A3 response element (nerve growth factor IB response element, NBRE, AAAGGTCA) located -3458 to -3268 upstream of its transcription start site. Using chromatin immunoprecipitation, we confirmed that NR4A3 binds to this site in the MAO-A promoter, with a 6-fold increase in interaction between NR4A3 and the MAO-A promoter in transgenic versus wild-type mice (figure 4F).

*Increased catecholamine catabolism in adipose tissue causes behavioral and cardiac alterations in AP2-NR4A3 mice.* We suspected that the highly reduced epinephrine observed in AP2-NR4A3 animals would produce other physiologic sequelae. While 24-hour energy expenditure was increased in transgenic animals versus wild-type, this difference was rendered not significant following normalization of energy expenditure measurements for body weight. Although we did not observe energy expenditure differences in a home-cage environment, AP2-NR4A3 did display reduced activity in an unfamiliar and stressful environment; in the open field test, transgenic animals had reduced center time \((P < 0.05)\) (Fig. 6A), reduced center distance \((P < 0.05)\), and increased side time \((P < 0.05)\) (Fig. 6B), with no difference in side distance. Because we observed no differences in serum corticosterone or 24-hour fecal corticosterone catabolites (Fig. 6C and D), we attribute these behavioral effects to decreased circulating epinephrine.

AP2-NR4A3 mice also displayed impaired myocardial dynamics. In the absence of anesthesia, AP2-NR4A3 exhibit highly reduced heart rate compared to
wild-type, as measured by tail-cuff plethysmography ($P < 0.001$) (Fig. 7A), without significant differences in systolic or diastolic blood pressure (AP2-NR4A3 mean systolic 102.6 mmHg, wild-type mean systolic 101.5 mmHg; C mean diastolic 85.6 mmHg, wild-type mean diastolic 85 mmHg). Echocardiogram measures are shown in Fig. 7B-F and Table 3. Following isoflurane anesthesia, AP2-NR4A3 heart rate did not differ from wild-type mice, as measured during echocardiography (NS) (Fig. 7B). Echocardiography indicated decreased left ventricular (LV) mass per body weight in transgenic versus wild-type mice (Fig. 7C). AP2-NR4A3 mice also displayed increased LV internal dimension during diastole ($P < 0.01$) and systole ($P < 0.05$) (measurements based on M-mode images) (Table 3), and increased LV endocardial volume during diastole ($P < 0.05$) (calculation based on B-mode measures) (Table 3) versus wild-type mice. In keeping with these observations, AP2-NR4A3 mice displayed increased LV volume during both diastole ($P < 0.01$) and systole versus control mice ($P < 0.05$) (calculation based on LV internal diameter M-mode measures) (Fig. 7D). When compared with wild-type, transgenic mice also displayed decreased interventricular septum thickness ($P < 0.05$) (Fig. 7E). Thus, compared to wild-type mice, AP2-NR4A3 mice exhibited decreased LV mass and interventricular septum thickness, along with increased LV volume, indicative of LV dilation. Lastly, AP2-NR4A3 mice had significantly decreased cardiac output per body weight compared to control mice ($P < 0.05$), which may be due to slightly decreased heart rates since stroke volume did not differ between transgenic and wild-type (Fig. 7F). Representative M-mode images are given in Figure 7G.
Table 3. High resolution echocardiography measurements

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-type (N=13)</th>
<th>AP2-NR4A3 (N=13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>36.49 ± 1.03</td>
<td>39.93 ± 0.78</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>107.7 ± 4.74</td>
<td>107.1 ± 4.51</td>
<td>NS</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>4.15 ± 0.06</td>
<td>4.41 ± 0.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>2.84 ± 0.08</td>
<td>3.18 ± 0.09</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LVID Trace CO (ml/min)</td>
<td>19.8 ± 1.3</td>
<td>18.34 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>LVID stroke volume</td>
<td>47.47 ± 2.34</td>
<td>48.19 ± 1.55</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.73 ± 0.03</td>
<td>0.69 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.05 ± 0.04</td>
<td>0.99 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW;d/LV mass (mm/mg)</td>
<td>0.007 ± 0.00</td>
<td>0.007 ± 0.00</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW;s/LV mass (mm/mg)</td>
<td>0.010 ± 0.00</td>
<td>0.009 ± 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>0.96 ± 0.05</td>
<td>0.86 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>IVS;d /LV mass (mm/mg)</td>
<td>0.009 ± 0.00</td>
<td>0.008 ± 0.00</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IVS;s / LV mass (mm/mg)</td>
<td>0.012 ± 0.00</td>
<td>0.012 ± 0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Endocardial area;d (mm2)</td>
<td>23.92 ± 0.38</td>
<td>26.03 ± 0.67</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Endocardial area;s (mm2)</td>
<td>15.82 ± 0.70</td>
<td>17.54 ± 0.80</td>
<td>NS</td>
</tr>
<tr>
<td>Endocardial volume;d (µl)</td>
<td>64.4 ± 1.8</td>
<td>74.5 ± 3.16</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Endocardial volume;s (µl)</td>
<td>33.3 ± 2.5</td>
<td>39.7 ± 3.14</td>
<td>NS</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>60.94 ± 2.05</td>
<td>57.87 ± 2.68</td>
<td>NS</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>32.55 ± 1.47</td>
<td>30.68 ± 1.84</td>
<td>NS</td>
</tr>
<tr>
<td>MV E (mm/s)</td>
<td>454.44 ± 32.54</td>
<td>551.61 ± 37.15</td>
<td>NS</td>
</tr>
<tr>
<td>MV A (mm/s)</td>
<td>282.97 ± 18.52</td>
<td>328.50 ± 13.44</td>
<td>NS</td>
</tr>
<tr>
<td>MV decel rate (mm/s2)</td>
<td>-20305 ± 1653</td>
<td>-26329 ± 2225</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MV decel time (ms)</td>
<td>20.54 ± 1.31</td>
<td>19.77 ± 0.91</td>
<td>NS</td>
</tr>
<tr>
<td>MV E/A</td>
<td>1.64 ± 0.10</td>
<td>1.69 ± 0.11</td>
<td>NS</td>
</tr>
</tbody>
</table>

LV: left ventricle; LVID;d and LVID;s: left ventricular dimension at diastole and systole; LVID Trace CO: left ventricular dimension trace cardiac output; LVPW;d and LVPW;s: posterior wall thickness at diastole and systole; IVS: interventricular
Reduced circulating epinephrine is not due to transgene expression in adrenal gland or hypothalamus. Because we observed highly decreased serum epinephrine with multiple physiologic consequences, we suspected transgene expression in the adrenal medulla or hypothalamus. Immunohistochemical staining for NR4A3 in adrenal glands was pronounced and we observed no significant differences between AP2-NR4A3 and wild-type mice (data not shown). We proceeded to assay for enzymes involved in adrenal epinephrine synthesis, storage, and catabolism via real-time rtPCR and observed no differences in transcription of the following: tyrosine hydroxylase, dopamine β-hydroxylase, phenylethanolamine N-methyltransferase, chromogranin, monoamine oxidases A and B, and catechol-O-methyltransferase. In order to determine whether the AP2 promoter might be active in hypothalamus, we also tested for endogenous expression of AP2 (adipocyte fatty acid binding protein, FABP4) in a panel of 9 tissues including hypothalamus. We found that hypothalamic expression of AP2 is either not detectable or approximately 500-fold lower than expression in gonadal fat (data not shown). We therefore conclude that the phenotype observed in AP2-NR4A3 mice is wholly attributable to changes in adipose tissue.
DISCUSSION

Our group has shown that NR4A3 overexpression in cultured 3T3-L1 cells leads to enhanced insulin-stimulated glucose uptake (11). We therefore made AP2-NR4A3 mice in order to observe physiological effects of NR4A3 overexpression in adipose tissue. We hypothesized that AP2-NR4A3 mice would display increased insulin sensitivity, increased glucose tolerance, and resistance to high fat diet-induced obesity and insulin resistance.

We were therefore surprised to observe that AP2-NR4A3 transgenic mice exhibit impaired glucose and insulin tolerance, decreased free fatty acids, and increased LDL when fed standard rodent diet. When AP2-NR4A3 mice are maintained on a high fat diet, glucose and insulin tolerance do not differ from wild-type. This may be because both AP2-NR4A3 and wild-type mice display severe obesity and insulin resistance when fed high fat diet; thus, high fat diet may have obscured the differences that we observed in chow-fed animals.

Importantly, circulating catecholamine levels are dramatically reduced in AP2-NR4A3 mice due to increased catecholamine degradation in adipose tissue.

Decreased adrenergic tone could account for elevated fasting glucose and poor glucose tolerance in transgenic mice. While α-adrenergic receptor agonism has been shown to decrease glucose-stimulated insulin secretion, β-adrenergic receptor agonism potentiates pancreatic insulin secretion under certain conditions (1, 7, 17, 18, 26, 29). Furthermore, decreased serum epinephrine may also account for decreased muscle insulin sensitivity in AP2-NR4A3 mice since proper adrenergic
tone may be required for optimal insulin signaling in skeletal muscle. In humans, long-term treatment (1-3 years) with certain β-blockers (pindolol, propanolol, metoprolol, or atenolol) causes decreased insulin sensitivity as assessed by hyperinsulinemic-euglycemic clamp (22). Additionally, treatment with dilevolol, a β-2 and β-3 agonist, improves insulin-stimulated glucose disposal in essential hypertension patients (13). Chronic (5-6 weeks) oral administration of clenbuterol, a specific β-2 adrenergic agonist, increases insulin-stimulated glucose disposal in normal and Zucker fatty rats (4, 27). The AP2 promoter has also been shown to drive transgene expression in perivascular cells of the skeletal muscle (20), and this mechanism could also contribute to perturbed insulin sensitivity in skeletal muscle. When these data are taken together with our observation that insulin sensitivity is decreased following reduced epinephrine, it appears likely that chronically decreased β-adrenergic signaling at skeletal muscle decreases insulin sensitivity. Additionally, we observed serum lipid abnormalities in AP2-NR4A3 mice. Since transgenic mice displayed decreased fasting free fatty acids, we were puzzled to observe no significant differences in lipolytic responses to isoproterenol or insulin in vitro or in vivo. This observation may be explained by down-regulation of the β-3 adrenergic receptor and concomitant up-regulation of the α-1 adrenergic receptor in AP2-NR4A3 animals. Indeed, increased expression of α-1 receptors partially reverses detriments in lipolytic response to norepinephrine in brown adipocytes (5). Furthermore, β-1/β-2/β-3 triple knockout mice display normal or elevated fasting free fatty acids (14). Thus, it may be concluded that while β-3 adrenergic receptor stimulation mediates the lipolytic response in healthy rodent
adipose tissue, chronic suppression of β-3 adrenergic receptor expression can be
counterbalanced by increased expression of other adrenergic receptors. AP2-
NR4A3 mice also display highly increased LDL-cholesterol, which may be explained
by decreased circulating epinephrine and subsequent decreases in the vascular
uptake of LDL. In rabbits, both epinephrine and norepinephrine enhance LDL
uptake into carotid artery walls (2, 34). Similarly, epinephrine increases LDL uptake
in rat aorta (3).

While AP2-NR4A3 mice displayed no significant differences in activity in a
home-cage environment, we did observe reduced activity in a stressful
environment, the open field test. Similar observations have been made following
propanolol delivery to wild-type mice; following tube restraint, propanolol-treated
mice exhibit increased time to emerge into an open field (36), as well as reduced
exploratory behavior in a Y-maze test (37). We observed no differences in fecal
corticosterone catabolites (an indicator of hypothalamic-adrenal axis function) or in
serum corticosterone following decapitation in the absence of anesthesia (an
indicator of hypothalamic-adrenal axis response to acute stress). Thus, the
alteration in response to a novel or stressful environment observed in AP2-NR4A3
mice is likely mediated by lack of adrenergic signaling in pertinent brain regions.

In support of the supposition that AP2-NR4A3 mice have chronically low
circulating catecholamines, these mice display altered cardiac function, with
decreased heart rate, and enlarged LVs, but without overt heart failure. The
chronotropic effect of β-adrenergic stimulation in heart is well described. In normal
animals, isoproterenol increases, while β-blockers decrease heart rate. However, β-
1/β-2 adrenergic receptor double knockout mice display normal blood pressure and heart rate under basal conditions, but exhibit reduced heart rate (versus wild-type) when challenged with isoproterenol (33). Thus, the highly decreased heart rate in AP2-NR4A3 during tail cuff plethsmography, but not during isoflurane anesthesia, may indicate a blunted chronotropic response to stress induced by human handling and constraint. It is also possible that the cardiac phenotype in AP2-NR4A3 transgenic mice is due to transgene expression in heart endothelial cells, with potential concurrent up-regulation of monoamine oxidase expression (20). This would be consistent with the observation that MAO-A mediates norepinephrine-induced hypertrophy in cultured cardiomyocytes independently of adrenergic receptor signaling, and that dominant-negative MAO-A mice exhibit decreased LV dimension without impaired LV function (16). It is also possible that changes in adrenergic receptor expression in the hearts of AP2-NR4A3 mice contribute to LV hypertrophy; in a context-dependent manner, α-1 adrenoreceptors stimulate LV hypertrophy (9).

It is possible that various aspects of the AP2-NR4A3 mouse phenotype are due to transgene expression outside of adipose tissue, skeletal muscle, or heart; the AP2 promoter is also active in mouse lung, developing sperm (20), and THP1 macrophage foam cells (10). Nonetheless, our data indicate that NR4A3 activates monoamine oxidase-A transcription in adipose tissue. It is noteworthy that the MAO-A gene also contains five intronic NR4A response elements that may act as transcriptional enhancers. In adipose tissue, MAO-A is up-regulated following delivery of the PPAR-γ agonist GW1929 (40), and in cultured adipocytes MAO-A
agonism induces glucose transport via GLUT4 translocation, independent of classical insulin signaling pathways (23, 39). Thus, chronic versus acute effects of MAO-A up-regulation may help to explain the discrepancy between our current in vivo findings and our previous in vitro findings, in which NR4A3 overexpression enhanced insulin-stimulated glucose transport via increased translocation of GLUT4 to the plasma membrane (9, 38). Given the importance of MAO-A activity in numerous diseases, it would be useful to determine whether NR4A3 modulates MAO-A transcription in other cell and tissue types. It is also possible that modulation of monoamine oxidases and COMT in adipose tissue may affect dose response to a variety of anti-hypertensive, psychiatric, and neurological drugs.

It is also possible that some aspects of the phenotype we observed were subtle due to large temporal and situational variation in NR4A3 protein. NR4A3 has been described as an “immediate early” gene, and many of our mouse adipose tissue blots (not shown) indicate a high degree of variability in protein levels in both transgenic and wild-type mice.

In conclusion, AP2-NR4A3 mice display a complex phenotype, including poor glucose tolerance and insulin resistance, together with a substantial decrease in circulating catecholamines. Figure 8 provides an overview of the physiological consequences of AP2-driven NR4A3 over-expression. The reduction in catecholamines in AP2-NR4A3 animals is associated with increased catecholamine degradation in adipose tissue. In our mice, NR4A3 over-expression in adipose tissue induced increased expression of catecholamine catabolizing enzymes, and we also demonstrated direct protein-DNA interaction between NR4A3 and the
MAO-A promoter. The decrease in catecholamines systemically impacts circulating lipids, cardiac function, and stress behavior.

ACKNOWLEDGEMENTS

The authors are extremely grateful to our undergraduate student assistant, Sasha T. Smith, for her sustained and meticulous collection of food intake and body weight data. We would also like to thank Dr. Douglas R. Moellering and Dr. Timothy R. Nagy for intellectual contributions.

We received research support from a number of UAB core facilities. As such, we would like to thank Dr. David Allison (UAB Nutrition Obesity Research Center), Dr. Barbara A. Gower and Maryellen Williams (UAB Diabetes Research and Treatment Center Human Physiology Core), Dr. Maria Johnson and Dr. Timothy R. Nagy (UAB Diabetes Research and Treatment Center Animal Physiology Core), Dr. Kevin A. Roth and Dr. Terry Lewis (UAB Neuroscience Molecular Detection Core, P30 NS47466), Dr. Elliot J Lefkowitz (Molecular and Genetic Bioinformatics Facility, 5UL1 RR025777), Dr. Thomas Van Groen (UAB Small Animal Behavioral Core, P30 NS47466).

GRANTS

This work was funded by NIDDK RO1 DK083562 (Garvey), a Department of Veterans Affairs Merit Review research award (Garvey), the UAB Diabetes Research Center, P60 DK-079626 (Garvey), NIH/NHLBI RO1 HL084611 (Bruemmer), ANDRO1 HL084611-S1 (Bruemmer).
DISCLOSURES

No conflicts of interest pertaining to this work, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.G.W., D.B., Y.F., and WTG conception and design of research; R.G.W., X.Z., L.T., E.B.H., H.S.H., Jiarong Liu, and Jian.Liu performed experiments. R.G.W., D.B., Q.Y., Y.F., and WTG interpreted results of experiments; R.G.W. prepared figures; R.G.W. and WTG drafted manuscript; R.G.W., Y.F., and WTG edited and revised manuscript; All authors approved final version of manuscript.

References


19. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanewise JS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ, Chamber Quantification Writing G, American
Society of Echocardiography's G, Standards C, and European Association of E. Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. Journal of the American Society of Echocardiography: official publication of the American Society of Echocardiography 18: 1440-1463, 2005.


**FIGURE LEGENDS**

Figure 1. Verification of AP2-NR4A3 transgene insertion and function. A: Schematic of the AP2-NR4A3 transgene. B: Transgene expression was verified by reverse transcriptase PCR. C and D: Western blot indicates that total NR4A3 protein is
increased in AP2-NR4A3 transgenic versus wild-type mice ($P < 0.05$, $N = 8$ AP2-NR4A3, 7 wild-type, normalized to β-actin). Analyses were performed using gonadal fat. WT=Wild-type, TG=AP2-NR4A3 transgenic. Error bars represent SEM. Figure 2. AP2-NR4A3 mice display metabolic syndrome when fed standard rodent diet. A: Transgenic mice weigh more than wild-type from 20 to 30 weeks of age (RMANOVA: genotype $P < 0.01$, time $P < 0.001$, time x genotype NS, post-hoc genotype $P < 0.05$ at 26, 27, 28, 29, and 30 weeks old). B: At age 31 weeks, fasting blood glucose is increased in AP2-NR4A3 mice ($P < 0.05$). C: 31-week old transgenic mice have impaired glucose tolerance (RMANOVA: genotype $P < 0.01$, time $P < 0.001$, time x genotype $P < 0.01$, post-hoc genotype $P < 0.05$ at 60 and 90 minutes). D: Fasting insulin is elevated in AP2-NR4A3 mice at age 32 weeks ($P < 0.05$). E: At 32 weeks of age, AP2-NR4A3 mice are insulin resistant, as assessed by ITT (RMANOVA: genotype $P < 0.05$, time $P < 0.001$, time x genotype $P < 0.05$, post-hoc genotype $P < 0.05$ at 30, 60 and 90 minutes). F: AP2-NR4A3 mice display decreased response to ITT, as assessed by AUC blood glucose following insulin challenge ($P < 0.05$). N = 17 AP2-NR4A3, 17 wild-type. Error bars represent SEM. Figure 3. At age 16-18 weeks, AP2-NR4A3 mice display no differences in in vivo lipolysis or anti-lipolysis, despite marked reductions in serum catecholamines. A: Following insulin delivery, serum norepinephrine, measured by HPLC, is reduced in AP2-NR4A3 mice ($P < 0.05$). B: Following insulin delivery, serum epinephrine, measured by HPLC, is dramatically reduced in AP2-NR4A3 mice ($P < 0.001$). C: Following insulin delivery, endogenous insulin levels do not differ between AP2-NR4A3 and wild-type mice. D: Following insulin or isoproterenol delivery, serum free fatty acids do not differ between AP2-NR4A3 and wild-type animals. N = 9 AP2-NR4A3, 9 wild-type. Error bars represent SEM. Figure 4. At age 16-18 weeks, serum epinephrine is highly reduced in AP2-NR4A3 mice due to enhanced epinephrine catabolism in adipose tissue. A: Serum epinephrine is drastically reduced in untreated AP2-NR4A3 mice ($P < 0.001$, N = 9 AP2-NR4A3, 9 wild-type). B: Epinephrine clearance is increased in cultured adipose tissue from AP2-NR4A3 versus wild-type animals ($P < 0.05$, N = 8 AP2-NR4A3, 8 wild-type). C: Genes for epinephrine catabolism enzymes are up-regulated in adipose tissue from AP2-NR4A3 mice. The β3-adrenergic receptor is downregulated while the α1-adrenergic receptor is up-regulated. Mean gene expression of catecholamine transporters is also increased in AP2-NR4A3 mice. MAO-A=monoamine oxidase A; MAO-B=monoamine oxidase B; COMT=catechol-O-methyltransferase; α1-AR= α1-adrenergic receptor; β3-AR= β3-adrenergic receptor; NET=extraneuronal norepinephrine transporter; OCT-1=organic cation transporter-1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Real-time PCR N = 11 AP2-NR4A3, 11 wild-type. D and E: There is a trend toward increased MAO-A protein (normalized to β-actin) in AP2-NR4A3 adipose tissue versus wild-type ($P = 0.1$, N = 5 AP2-NR4A3, 4 wild-type). F: chromatin immunoprecipitation results indicate that NR4A3 binds to the MAO-A promoter, with a 6-fold increase in interaction between NR4A3 and the MAO-A promoter in transgenic versus wild-type mice (N=2 AP2-NR4A3, 2 wild-
type). PCR primers flank a true NBRE, -3458 to -3268 upstream of the MAO-A transcription start site. Analyses were performed using gonadal fat. Error bars represent SEM.

Figure 5. Schematic of epinephrine degradation in adipose tissue. COMT=Catechol-O-methyltransferase, MAO=Monoamine oxidases A & B, NET=Norepinephrine transporter, OCT=Organic cation transporter. Epinephrine is transported into cells by NET and OCT. It may be converted to dihydroxymandelic acid by mitochondrial MAOs, and then converted to vanillylmandelic acid by membrane-bound COMT. Alternately, intracellular epinephrine may be converted to metanephrine by cytosolic COMT, and then converted to vanillylmandelic acid by MAOs. Vanillylmandelic acid is released into the circulation and excreted by the kidneys. Adipose tissue also secretes Renalase, a soluble amine oxidase, into the circulation.

Figure 6. AP2-NR4A3 exhibit behavioral alterations that cannot be explained by differences in corticosterone. A: In the open field test, 16-week old AP2-NR4A3 mice spend less time in the center of the field ($P < 0.05$) and B: travel less distance in the center of the field ($P < 0.05$, $N = 8$ AP2-NR4A3, 8 wild-type). C: At age 14-18 weeks, serum corticosterone does not differ between AP2-NR4A3 and wild-type mice. Serum corticosterone did not correlate to order of sacrifice ($N = 9$ AP2-NR4A3, 9 wild-type). D: In 13-17-week old animals, 24-hour production of corticosterone, assessed by measurement of corticosterone metabolites in feces, does not differ between AP2-NR4A3 and wild-type mice ($N = 13$ AP2-NR4A3, 13 wild-type). Error bars represent SEM.

Figure 7. At age 24-26 weeks, AP2-NR4A3 mice have abnormal cardiac function. A: Heart rate, measured by tail-cuff plethysmography in awake mice, is robustly decreased in AP2-NR4A3 mice versus wild-type ($P < 0.001$, each measurement represents the mean of approximately 45 observations per animal, $N = 12$ AP2-NR4A3, 12 wild-type). B: During echocardiography, mean heart rate remains decreased in anesthetized AP2-NR4A3 versus wild-type animals, but this difference is not significant. C: Compared to control mice, AP2-NR4A3 mice have decreased ratio of LV mass to body weight ($P < 0.05$). D: As calculated from M-mode echocardiography images, AP2-NR4A3 mice have increased LV volume during both diastole ($P < 0.01$) and systole ($P < 0.05$) versus wild-type mice. E: Systolic interventricular septum thickness is decreased in AP2-NR4A3 mice compared to wild-type ($P < 0.05$). F: Compared to control mice, AP2-NR4A3 mice display decreased LVID trace cardiac output when adjusted for body weight ($P < 0.05$). G: Representative M-mode images from wild-type and AP2-NR4A3 mice. Echocardiography $N = 13$ AP2-NR4A3, 13 wild-type. Error bars represent SEM.

Figure 8. Overview of AP2-NR4A3 phenotype. Adipocyte-specific NR4A3 over-expression induces increased expression of enzymes that degrade catecholamines, including monoamine oxidases A and B, catechol-O-methyltransferase, and renalase. Increased catecholamine degradation in adipose tissue causes
chronically decreased circulating epinephrine, with pleiotropic physiological consequences. Chronic epinephrine reduction leads to poor glucose tolerance, insulin resistance, increased LDL, decreased cardiac chronotropy, LV dilation, and decreased activity in a stressful environment.
Figure 1

A

![Diagram showing the AP2 promoter, NR4A3, and PolyA regions with restriction enzyme sites KpnI, HindIII, and NotI, and the ATG start codon.]

B

![Image showing gel electrophoresis with markers for WT, TG, WT, and TG samples.]

C

![Image showing a Western blot with bands for NR4A3 at 68 kDa and beta-actin at 42 kDa. The image includes wild-type and AP2-NR4A3 samples.]}

D

![Bar graph showing NR4A3/β-actin optical density/mm² comparison between AP2-NR4A3 and wild-type samples. The graph includes error bars and a star indicating a significant difference.]
Figure 2

A. Body weight (g) over weeks for AP2-NR4A3 and Wild-type, with statistical significance indicated by "P<0.01, RMANOVA".

B. Blood glucose levels (mg/dL) with a significant difference indicated by "*".

C. Blood glucose levels (mg/dL) over time (minutes) showing a significant difference indicated by "P<0.01, RMANOVA".

D. Serum insulin levels (mg/ml) with a significant difference indicated by "*".

E. Blood glucose levels (mg/dL) over time (minutes) with a significant difference indicated by "P<0.05, RMANOVA".

F. Area under the curve for blood glucose levels (mg/dL) with a significant difference indicated by "*".
Figure 5

Epinephrine degradation

COMT=Catechol-O-methyltransferase
MAO=Monoamine oxidases A & B
NET=Norepinephrine transporter
OCT=Organic cation transporter

Vanillylmandelic acid (VMA)

Mitochondria
MAO

COMT

Metanephrine

Dihydroxy-mandelic acid

Renalase

Epinephrine

OCT

NET
Figure 8

↑ NR4A3

↑ Catecholamine degradation enzymes in adipose tissue
  - ↑ Monoamine oxidase A
  - ↑ Monoamine oxidase B
  - ↑ Catechol-O-methyltransferase
  - ↑ Renalase

↑ Catecholamine clearance in adipose tissue

↓ Circulating catecholamines especially epinephrine

- Pancreas: ↓ Glucose tolerance
- Skeletal muscle: ↓ Insulin sensitivity
- Liver, Vasculature: ↑ LDL
- Heart: ↓ Chronotropy, ↑ LV dilation
- Behavior: ↑ Anxiety