

# Antiobesity Effect of a Small Molecule Repressor of ROR $\gamma$ <sup>S</sup>

Mi Ra Chang, Yuanjun He, Tanya M. Khan, Dana S. Kuruvilla, Ruben Garcia-Ordonez, Cesar A. Corzo, Thaddeus J. Unger, David W. White, Susan Khan, Li Lin, Michael D. Cameron, Theodore M. Kamenecka, and Patrick R. Griffin

*Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, Florida (M.R.C., Y.H., T.M.K., D.S.K., R.G.-O., C.A.C., S.K., L.L., M.D.C., Th.M.K., P.R.G.); and Ember Therapeutics, Watertown, Massachusetts (T.J.U., D.W.W.)*

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## ABSTRACT

The orphan nuclear receptor ROR $\gamma$  is a key regulator for T helper 17 (T<sub>H</sub>17) cell differentiation, which regulates metabolic and circadian rhythm genes in peripheral tissues. Previously, it was shown that the small molecule inverse agonist of ROR $\gamma$  SR1555 [1-(4-((4'-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-[1,1'-biphenyl]-4-yl)methyl)piperazin-1-yl) ethanone] suppressed T<sub>H</sub>17 differentiation and stimulated induced T regulatory (iTreg) cells. Here, we show that treatment of cultured pre-adipocytes with SR1555 represses the expression of ROR $\gamma$  while leading to increased expression of FGF21 and adipoQ. Chronic administration of SR1555 to obese diabetic mice resulted in a modest reduction in food intake accompanied with significant reduction in

fat mass, resulting in reduced body weight and improved insulin sensitivity. Analysis *ex vivo* of treated mice demonstrates that SR1555 induced expression of the thermogenic gene program in fat depots. Further studies in cultured cells showed that SR1555 inhibited activation of hormone-sensitive lipase and increased fatty acid oxidation. Combined, these results suggest that pharmacological repression of ROR $\gamma$  may represent a strategy for treatment of obesity by increasing thermogenesis and fatty acid oxidation, while inhibition of hormone-sensitive lipase activity results in a reduction of serum free fatty acids, leading to improved peripheral insulin sensitivity.

## Introduction

The percentage of the global population categorized as overweight or obese has increased dramatically over the last few decades. This trend is predicted to continue as developed and developing nations increasingly adopt more sedentary lifestyles and gain easier access to high calorie diets. The metabolic syndrome is associated with obesity, and patients with this syndrome are at a significant increased risk of suffering from cardiovascular disease and stroke (Moller and Kaufman, 2005). There are two major underlying drivers for the development of metabolic syndrome: excess adiposity (obesity) and type 2 diabetes mellitus (Grundy et al., 2005).

Type 2 diabetes mellitus is a chronic metabolic disorder that results in part by the inability of the body to respond adequately to circulating insulin, a state of insulin resistance. In the obese state, free fatty acids (FFAs) are elevated in plasma and in all insulin responsive organs including skeletal muscle, liver, and endothelial cells. As such, elevated FFAs are linked to the development of the metabolic syndrome. Obesity is also closely associated with a low-grade state of inflammation characterized by elevated pro-inflammatory cytokines in blood and tissues (Tataranni and Ortega, 2005).

Treatments for metabolic syndrome included modification of diet and increased exercise (Grundy et al., 2004). However, pharmacologic intervention is typically required because weight loss and exercise often are not sufficient due to poor compliance and confounding genetic factors (Bouchard, 1988; Moller et al., 1996). Members of the nuclear receptor (NR) superfamily are ligand-controlled transcription factors that regulate a wide range of metabolic, endocrine, and immunologic functions, and this protein superfamily has proven to be a rich source of targets for the development of therapeutics for a wide range of human diseases including inflammation and diabetes. A subset of NRs is classified as orphan receptors due

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**ABBREVIATIONS:** CLAMS, comprehensive laboratory animal monitoring system; DIO, diet-induced obesity; FBS, fetal bovine serum; FFA, free fatty acid; HSL, hormone-sensitive lipase; NR, nuclear receptor; PBS, phosphate-buffered saline; ROR, retinoic acid receptor-related orphan receptor; SR1001, *N*-[4-methyl-5-[[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]amino]sulfonyl]-2-thiazolylacetamide; SR1078, *N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-4-(trifluoromethyl)benzamide; SR1555, 1-(4-((4'-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-[1,1'-biphenyl]-4-yl)methyl)piperazin-1-yl) ethanone; SR2211, 1,1,1,3,3,3-hexafluoro-2-(4'-((4-(pyridin-4-ylmethyl)piperazin-1-yl)methyl)-[1,1'-biphenyl]-4-yl)propan-2-ol; SR3335, *N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-2-thiophenesulfonamide; SVF, stromal vascular fraction; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; T<sub>H</sub>17, T helper 17; Treg, T regulatory; TR-FRET, time-resolved fluorescence resonance energy transfer; VO<sub>2</sub>, volume of oxygen.

to lack of a characterized or agreed upon endogenous ligand (Kliewer et al., 1999). The retinoic acid receptor-related orphan receptor (ROR) NR1F subfamily was identified based on sequence similarities to the retinoic acid and retinoid X receptors (Becker-André et al., 1993; Giguère et al., 1994). The T cell-specific  $\gamma$  isoform, ROR $\gamma$ t, has been the focus of considerable attention due to its role in the development of T helper 17 (T<sub>H</sub>17) cells and in the pathology of autoimmune disease. While ROR $\gamma$ t is highly expressed in immune cells and thymus, ROR $\gamma$  variants are expressed in the liver, skeletal muscle, adipose tissue, and kidney (Jetten, 2009), and its expression can be induced in macrophages during acute inflammatory responses (Barish et al., 2005; Gu et al., 2008; Chang et al., 2014). Furthermore, genetic deletion of ROR $\gamma$  results in profound effects on adipose depots with increased adipocyte numbers, yet reduced hypertrophy accompanied by improved insulin sensitivity. These ROR $\gamma$ -deficient mice were shown to be resistant to diet-induced insulin resistance (Meissburger et al., 2011).

Recently, synthetic modulators of the ROR subfamily have been described (Kojetin and Burris, 2014; Marciano et al., 2014), including a series of reports on the development of potent and selective inverse agonists of ROR $\gamma$ . These studies demonstrate their utility in reducing the severity of inflammation in mouse models of multiple sclerosis (experimental autoimmune encephalomyelitis) (Huh et al., 2011; Solt et al., 2011), and rheumatoid arthritis (collagen-induced arthritis) (Chang et al., 2014). One such compound, SR1555 [1-(4-((4'-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-[1,1'-biphenyl]-4-yl)methyl)piperazin-1-yl) ethanone], was previously shown to inhibit T<sub>H</sub>17 cell development while increasing the frequency of T regulatory (Treg) cells (Solt et al., 2012). Thus, SR1555 was evaluated in the collagen-induced arthritis

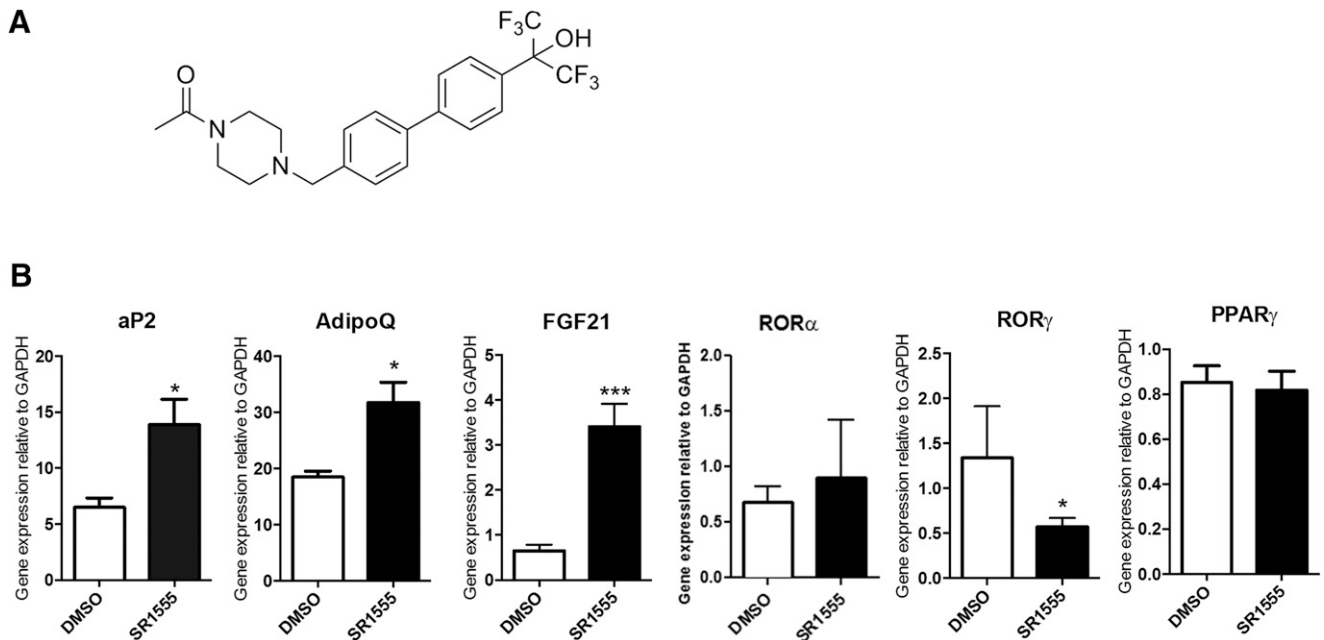
mouse model (Chang et al., 2014). In addition to observing a reduction in joint inflammation, effects on body weight and adiposity were noted. Based on this observation SR1555 was evaluated in the diet-induced obesity (DIO) murine model of obesity and type 2 diabetes mellitus, and the results of these studies are presented here.

## Materials and Methods

**Chemicals.** SR1555 (Fig. 1A) was synthesized as previously described (Solt et al., 2012).

**LanthaScreen Time-Resolved Fluorescence Resonance Energy Transfer Competitive Binding Assay for PPAR $\gamma$ .** The PPAR $\gamma$  competitive binding assay (Invitrogen, Carlsbad, CA) was performed according to the manufacturer's protocol. A mixture of 0.5 nM GST-PPAR $\gamma$  LBD, 5 nM Tb-GST-antibody, 5 nM fluormone Pan-PPAR Green, and serial dilutions of the compound beginning at 10  $\mu$ M downward was added in 384-well low-volume plates (Greiner Bio-One, Monroe, NC) to a total volume of 18  $\mu$ l (2% dimethylsulfoxide in all wells). Dimethylsulfoxide at 2% concentration was used as a no-ligand control. All dilutions were made in time-resolved fluorescence resonance energy transfer (TR-FRET) PPAR assay buffer. Experiments were performed in triplicate and incubated for 2 hours in the dark before analysis in a PerkinElmer ViewLux Ultra HTS Microplate Reader (PerkinElmer, Waltham, MA). The TR-FRET signal was measured by excitation at 340 nm with emission at 520 nm for fluorescein and 490 nm for terbium. Data were plotted as the TR-FRET ratio 520/490 nm using the GraphPad Prism software (GraphPad Software, La Jolla, CA).

**Animals.** Seventeen-week-old male C57BL/6 DIO mice were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures were approved and conducted in accordance with the Scripps Florida Institutional Animal Care and Use Committee. Mice were placed on a high-fat diet (60% kcal derived from fat) for 7 weeks until they reached an average weight of  $\sim$ 40 g. Prior to and during



**Fig. 1.** SR1555 impacts adipogenic markers. (A) Chemical structure of SR1555 and (B) gene expression analysis in differentiated adipocytes. 3T3-L1 cells were grown to confluence, and then incubated with dexamethasone, insulin, and isobutylmethylxanthine for 2 days to induce differentiation. Cells were then treated with 20  $\mu$ M SR1555 and insulin for 5 days, after which the expression of thermogenic genes and several nuclear receptors were analyzed by quantitative polymerase chain reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used for normalization. Values are the mean  $\pm$  S.E.M. of three samples per group. \* $P$  < 0.05, \*\*\* $P$  < 0.001 versus dimethylsulfoxide (DMSO), as determined using Student's unpaired  $t$  test.

compound administration mice were maintained on a high-fat diet. Animals were sham dosed with vehicle for 3 days prior to compound administration. SR1555 was administered intraperitoneally twice a day at 10 or 20 mg/kg or by oral gavage at 20 mg/kg once a day. Body weight and food intake were monitored daily. Pre- and post experiment whole-body composition analysis was performed on vehicle only-treated and SR1555-treated mice using NMR (Minispec mq 7.5 NMR analyzer; Bruker Optics, Billerica, MA). Blood samples were collected for plasma cholesterol, triglyceride, FFA, and insulin measurements. For insulin tolerance tests, mice were injected (intraperitoneally) with 10 mg/kg SR1555 for 18 days, and fasted 6 hours before intraperitoneal injection of insulin (0.75 U/kg, Sigma-Aldrich, St. Louis, MO). Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes using a glucometer (LifeScan, Milpitas, CA). Mice were allowed to recover from the stress associated with the insulin tolerance test for 7 days prior to performing the glucose tolerance test. Here, mice were fasted overnight before injection of 2 g/kg D-glucose intraperitoneally, and blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes.

**In Vitro Adipocyte Differentiation.** 3T3-L1 and C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL/Life Technologies, Grand Island, NY). Twenty-four hours postconfluence, the 3T3-L1 cells were differentiated with isobutylmethylxanthine (0.5 mM), dexamethasone (1  $\mu$ M), insulin (10  $\mu$ g/ml), and 10% FBS for 48 hours. After induction of adipogenesis, the cells were maintained with insulin (10  $\mu$ g/ml) and 20  $\mu$ M of SR1555 for 3 or 4 days. The stromal vascular fraction (SVF) within adipose tissue was isolated from the epididymal adipose tissue of C57BL/6 mice to avoid contamination from nonadipose tissues as previously described (Koh et al., 2007). To exclude blood contamination in the adipose tissue, systemic perfusion with heparinized phosphate-buffered saline (PBS) was performed before harvesting or washing isolated adipose tissues with PBS. Then, the adipose tissues were incubated in Hanks' balanced salt solution (Sigma-Aldrich) containing 0.2% collagenase type 2 for 60 minutes at 37°C with constant shaking. After inactivation of collagenase activity with 10% FBS containing Dulbecco's modified eagle medium, the cell suspension was filtered through a 40  $\mu$ m nylon mesh (BD Biosciences, San Jose, CA) followed by centrifugation at 420g for 5 minutes. Floating adipocytes and supernatant were removed from the SVF pellet. The SVF pellet was washed and resuspended in sterilized PBS.

**Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was extracted from 3T3-L1 cells or tissues using TRIzol reagent (Invitrogen). The RNA was reverse transcribed using the ABI reverse transcription kit (Applied Biosystems/ThermoFisher Scientific, Waltham, MA). Quantitative polymerase chain reactions were performed with SYBR green fluorescent dye using an ABI9300 polymerase chain reaction machine. Relative mRNA expression was determined by the  $\Delta\Delta$ -Ct method normalized to glyceraldehyde-3-phosphate dehydrogenase levels. The sequences of primers used in this study are found in Supplemental Fig. 1.

**MitoTracker Fluorescence-Activated Cell Sorting Quantification.** C2C12 cells were washed with PBS, trypsinized, and incubated at 37°C for 20 minutes with 100 nM MitoTracker Green FM and Red GM dyes (Molecular Probes, Eugene, OR). The MitoTracker Green probe preferentially accumulates in mitochondria, allowing estimation of mitochondrial content. The MitoTracker Red probe is a red-fluorescent dye that stains mitochondria in living cells and its accumulation is dependent on the membrane potential (Molecular Probes). Samples were washed three times in PBS and subjected to flow cytometric analysis on a BD LSRII instrument (Becton Dickinson, San Jose, CA). The results were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Oxygen Consumption.** Immortalized SVF cells were seeded at  $1 \times 10^4$  cells per well in a differentiation medium containing Dulbecco's modified Eagle's medium with 10% FBS on Seahorse XF96 well culture plates (Seahorse Bioscience, North Billerica, MA).

Cellular oxygen consumption rate was measured using the Seahorse XF96 at 4 to 5 days differentiation with 20  $\mu$ M SR1555. The concentration of fatty acid (oleic and linoleic acids), oligomycin, carbonilcyanide p-trifluoromethoxyphenylhydrozone, and rotenone were 1 mg/ml, 10 mg/ml, 6 mM, and 3 mM, respectively.

**Resting Whole-Body Metabolic Parameters.** The daily average whole-body volume of oxygen (VO<sub>2</sub>) (ml/kg) per hour, and cage activity (movement counts) were measured in DIO mice using a comprehensive laboratory animal monitoring system (CLAMS) (Oxymax series; Columbus Instruments, Columbus, OH). Each sealed chamber (CLAMS unit) is equipped with an O<sub>2</sub> electrochemical sensor, a CO<sub>2</sub> infrared sensor, and infrared beam activity sensors. The units allow the VO<sub>2</sub> consumed (ml/kg) per hour to be measured. Infrared beam interruptions in both horizontal (X) and vertical (Z) directions were measured to quantify the motor activity of mice in both directions. Total horizontal beam breaks were summed to provide the total horizontal activity count. Two or more consecutive horizontal beam breaks were recorded as the ambulatory activity count. All vertical beam breaks were summed to provide the total vertical activity count. The CLAMS studies began after animals were acclimated to the metabolic chambers and VO<sub>2</sub> data were collected at 16 minute intervals over a 5 day period under a consistent environmental temperature (22°C). During these studies, mice were allowed free access to food and water.

**Western Blot Analysis.** Primary antibodies against phosphorylated hormone-sensitive lipase (HSL) (S660), total HSL, and  $\beta$ -actin were purchased from Cell Signaling Technology (Danvers, MA). A rabbit fast western blotting kit (Pierce/Thermo Scientific, Rockford, IL) was used for detection of primary antibody.

**Plasma Concentration of FGF21 and Insulin.** Blood samples were collected in heparin tubes on ice and centrifuged at 4°C. Plasma FGF21 and insulin levels were determined by a specific mouse enzyme-linked immunosorbent assay (Millipore, Billerica, MA) following the manufacturer's instructions.

**Plasma Lipid Profile.** Blood samples were collected in heparin tubes on ice and centrifuged at 4°C. Cholesterol, triglyceride, and FFA were quantitated using a Cobas c311 clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland).

**Pharmacokinetics.** The oral pharmacokinetic profile of SR1555 was determined in male C57Bl6 mice. SR1555 was formulated at a concentration of 1 mg/ml in 15% Cremophore EL (Sigma-Aldrich) in water and dosed by oral gavage to three mice at a final dose of 20 mg/kg. Blood was collected in lithium-heparin-coated capillary tubes using a microsampling technique at 15, 30, 60, 120, 240, 360, 480, and 1440 minutes and plasma was generated using standard centrifugation methods. Plasma concentrations were determined via liquid chromatography-tandem mass spectrometry on an ABSciex 5500 (Sciex, Framingham, MA). SR1555 was detected using the mass transition 461  $\rightarrow$  333, and concentrations were determined by comparison with a 9-point standard curve between 2 and 2000 ng/ml prepared in mouse plasma. Pharmacokinetic analysis was done with WinNonLin (Pharsight/Cetara, Princeton, NJ) using a noncompartmental model. All procedures described are covered under existing protocols and have been approved by the Scripps Florida Institutional Animal Care and Use Committee to be conducted in the Scripps Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

**Statistical Analysis.** The data are reported as the mean  $\pm$  S.E.M. Statistical significance was evaluated using the Student's unpaired *t* test. *P* values less than 0.05 were considered significant.

## Results

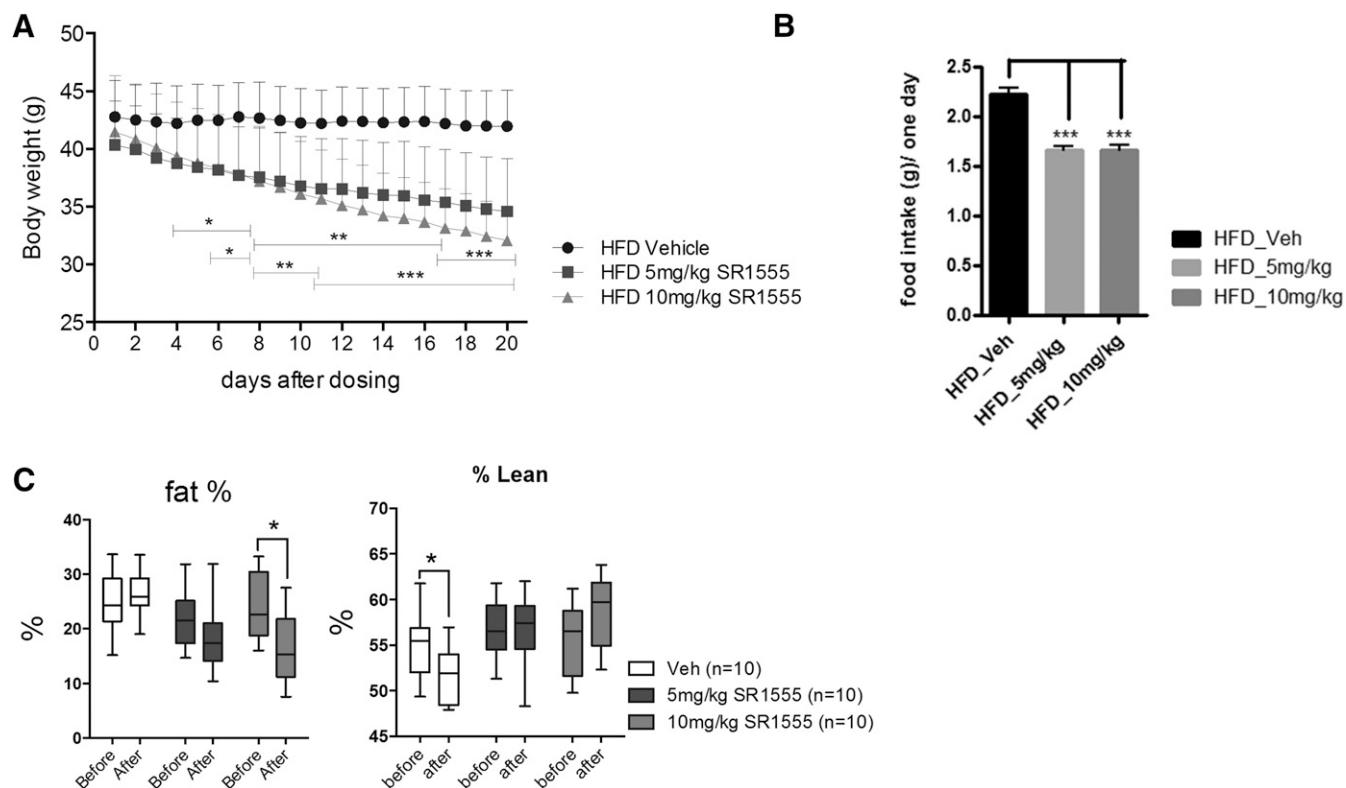
**SR1555 Alters Adipogenesis.** Previously, SR1555 (Fig. 1A) was shown to be a ROR $\gamma$ -specific inverse agonist that inhibited T<sub>H</sub>17 cell development and increased the Treg cell population (Solt et al., 2012). Because genetic deletion of ROR $\gamma$  results in profound effects on adipose tissue we investigated the

effects of SR1555 on adipogenesis using differentiated 3T3L1 cells. Treatment of these cells with SR1555 resulted in decreased expression of ROR $\gamma$  (Fig. 1B). Consistent with observations from ROR $\gamma$ -depleted mice, SR1555 treatment led to increased expression of *FABP4* (aP2) and *adiponectin* (*adipoQ*); however, it had no effect on the expression of PPAR $\gamma$ , a key adipogenic regulator. Interestingly, the expression of *FGF21* was increased in SR1555-treated 3T3L1 adipocytes. *FABP4* is a PPARG (*NR1C3*) target gene. Therefore, we tested the ability of SR1555 to bind to this NR. Consistent with results from broad selectivity profiling, and as shown in Supplemental Fig. 2A, in a TR-FRET competitive binding assay SR1555 was not able to displace labeled ligand at all concentrations tested. Furthermore, SR1555 was not able to transactivate the expression of luciferase in a reporter gene assay at all concentrations tested (Supplemental Fig. 2B).

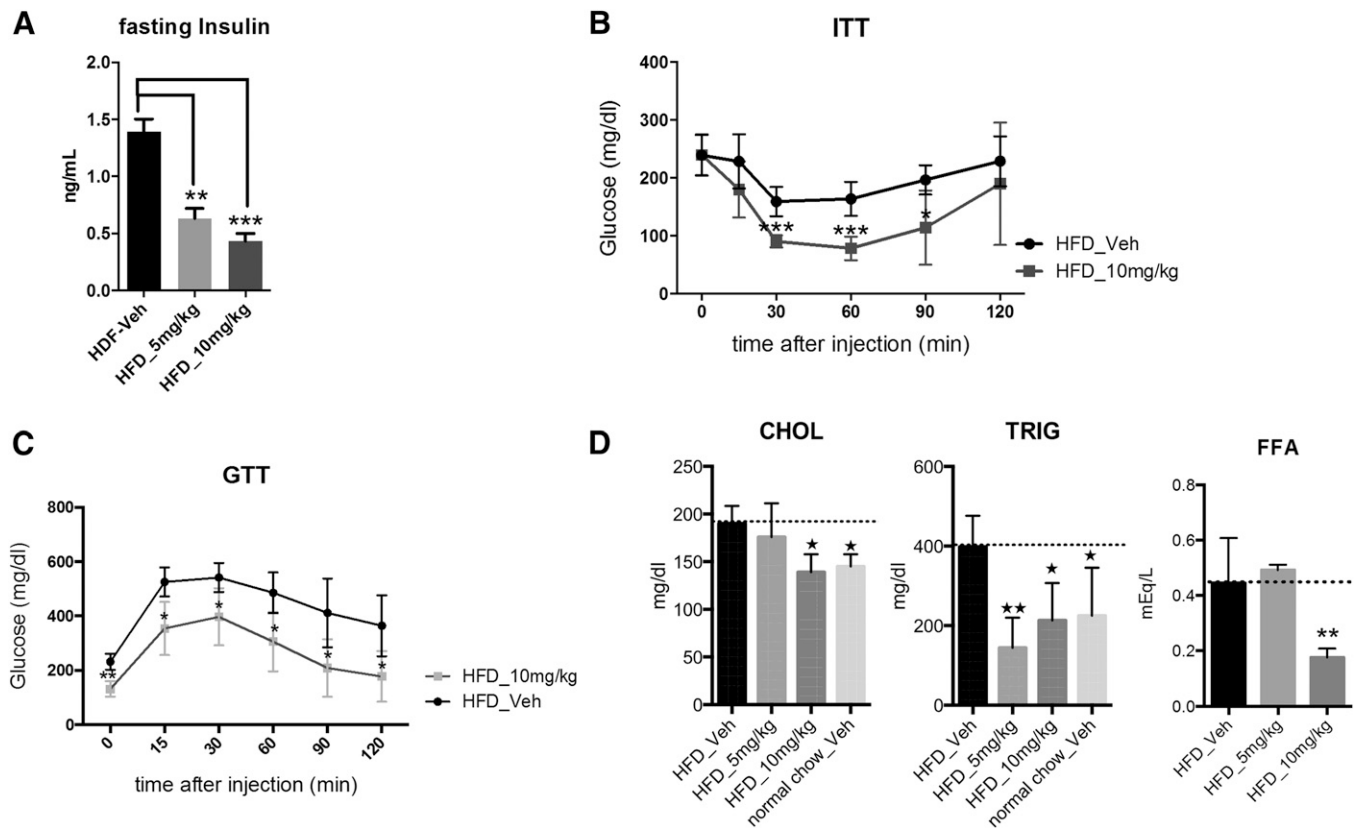
ROR $\gamma$ -deficient mice are resistant to obesity and insulin resistance (Meissburger et al., 2011). Based on these observations, we sought to determine the effects of pharmacological repression of ROR $\gamma$  in the DIO mouse model of obesity and diabetes. Mice were fed a high-fat diet until reaching obesity (average weight of ~40 g). DIO mice were then treated with vehicle (15% Cremophor, i.p.) for 3 days prior to initiating administration of SR1555. As shown in Supplemental Fig. 3, bolus administration of SR1555 to mice at 5 and 10 mg/kg i.p., and 20 mg/kg by oral gavage afforded plasma concentrations of the compound 4 hours postadministration in excess of 4, 8, and 16  $\mu$ M, respectively. The calculated pharmacokinetic

parameters are presented in Supplemental Fig. 4. With the intent of only partially repressing ROR $\gamma$  and based on the plasma exposure from the pharmacokinetic studies shown in Supplemental Fig. 3, SR1555 was administered intraperitoneally to obese mice at 5 or 10 mg/kg twice a day. Body weight and food intake were recorded daily. Following 20 days of compound administration, DIO mice receiving vehicle only maintained body weight but animals treated with SR1555 at 5 mg/kg had lost 15% of their body weight (Fig. 2A). SR1555 demonstrated a dose-dependent impact on body weight because mice administered compound at 10 mg/kg lost an additional 8% body weight (23% loss). Compound treatment did cause a modest reduction in food intake by DIO mice compared with control animals (Fig. 2B). However, no change in food intake was observed in lean mice, suggesting SR1555 is not driving weight loss by systemic toxicity. It is possible that SR1555 treatment results in an improvement in leptin sensitivity.

Whole-body composition of DIO mice was analyzed using NMR prior to the start of vehicle or compound administration and again at the conclusion of the study. As shown in Fig. 2C, lean mass was unaffected by SR1555 treatment; however, there was a statistically significant loss of fat mass in the 10 mg/kg SR1555-treated DIO mice. On day 18 of treatment, mice were fasted for 8 hours and then subjected to an insulin tolerance test. As shown in Fig. 3A fasting insulin was significantly reduced in both the 5 and 10 mg/kg arms of the study. Figure 3B illustrates that glucose disposal was improved with a quick decline in plasma glucose 15 minutes



**Fig. 2.** SR1555 administration mice are resistant to obesity. (A) Body weight of high-fat diet (HFD) controls and SR1555-treated obese mice, (B) food intake, and (C) whole-body NMR. Twenty-one week old DIO mice were administered SR1555 intraperitoneally at 5 or 10 mg/kg for 20 days. Body weight and food intake were monitored daily. Percentage fat was measured just prior to the start of compound administration and again after 17 days of compound administration. Values are the mean  $\pm$  S.E.M. of 10 samples per group. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 versus vehicle or before start dosing, by Student's unpaired  $t$  test. Veh, vehicle.



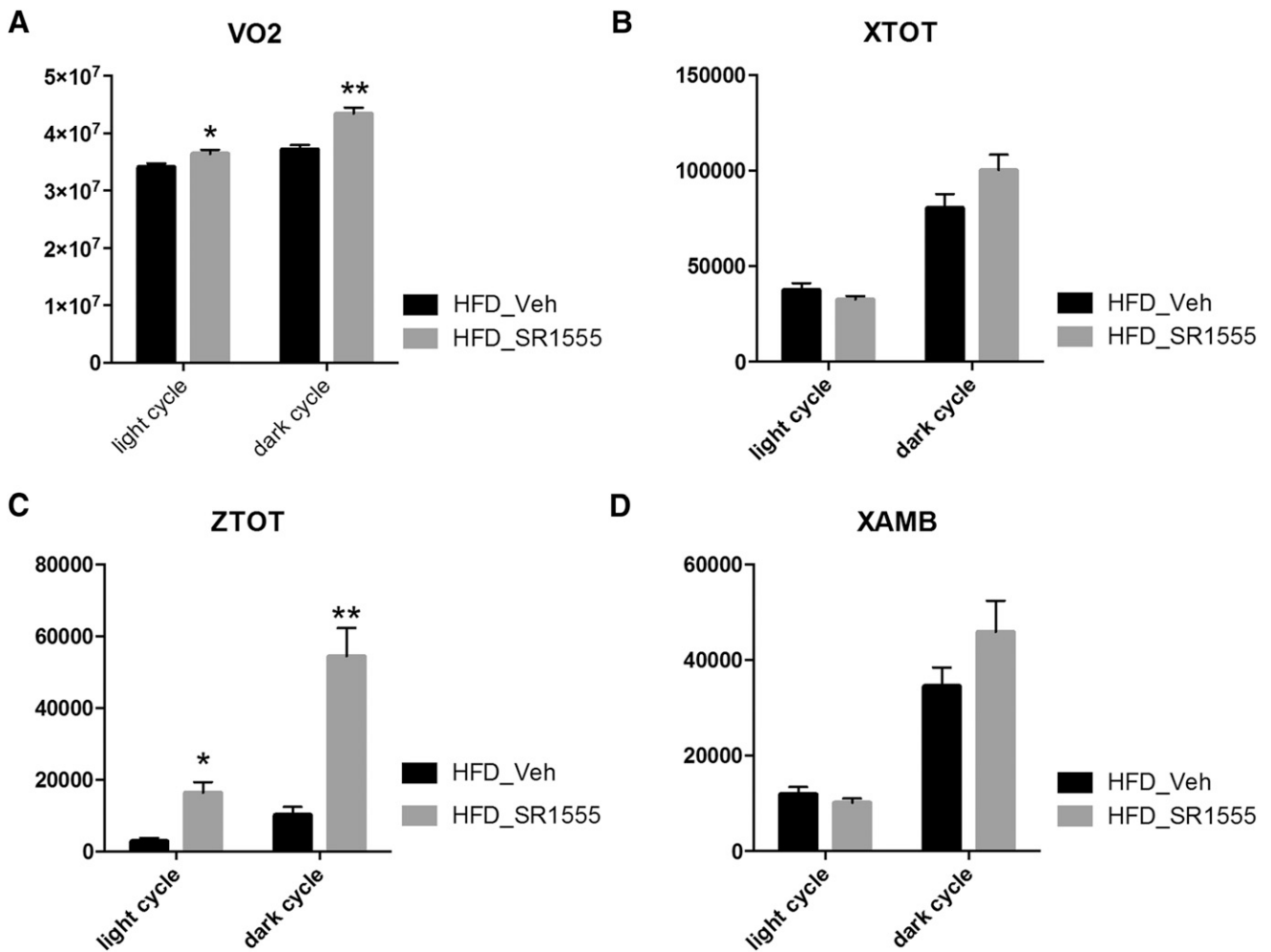
**Fig. 3.** Metabolic parameters in DIO mice were improved by SR1555 administration. (A) Cholesterol (CHOL), triglyceride (TRIG), and FFAs were measured in SR1555-treated DIO mice after 20 days dosing. (B) Fasting insulin and insulin (0.75 U/kg) tolerance test (ITT,  $n = 10$ ) were measured on DIO mice and (C) glucose tolerance test (GTT) performed 7 days following last dose of SR1555. Values are the mean  $\pm$  S.E.M. of 10 samples per group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus vehicle, by Student's unpaired  $t$  test. Veh, vehicle.

post insulin administration in the 10 mg/kg arm of the study. Statistically significant differences in plasma glucose were observed at 30, 60, and 90 minutes post insulin administration. Following the insulin tolerance test, compound administration was terminated and mice were allowed to recover from the stress of fasting. At 7 days post compound administration a glucose tolerance test was performed to determine if SR1555 treatment results in improved disposal of plasma glucose in response to a bolus intake of a glucose solution. As shown in Fig. 3C, DIO mice treated with SR1555 retained improved insulin sensitivity 7 days post compound treatment. Analysis of plasma lipids at this time point showed that SR1555 treatment reduced cholesterol and triglyceride levels below vehicle only-treated mice and to a level similar to that in control lean mice maintained on a normal chow diet (Fig. 3D). The FFA levels were also reduced in SR1555-treated animals (Fig. 3D). Although SR1555 increased expression of *FGF21* in adipocytes, compound treatment in DIO mice did not result in an increase in plasma *FGF21* (data not shown) secreted from the liver.

We next investigated whether whole-body energy expenditure and ambulatory activity were impacted by SR1555 treatment. DIO mice were acclimated to single animal housing for 7 days prior to the start of this study. SR1555 was then administered by oral gavage at 20 mg/kg for 7 days before placing the animals in CLAMS chambers. Once inside the CLAMS chambers, mice were administered SR1555 orally each day at 20 mg/kg and metabolic parameters and activity were evaluated over a 7 day period.

As shown in Fig. 4A, oxygen consumption ( $VO_2$ ) in compound-treated DIO mice was increased during both the light and dark cycles compared with the vehicle only-treated group. Furthermore, body temperature was significantly increased in SR1555-treated mice (Supplemental Fig. 5, A and B). No difference in the total horizontal activity count and ambulatory activity count was observed when comparing SR1555-treated mice to vehicle only-treated mice; however, the total vertical activity count was increased in compound-treated DIO mice (Fig. 4, B–D). It is possible that this increase in vertical activity may reflect increased exploration of the environment (attention), cognitive enhancement, or perhaps anxiety. Regardless, the data obtained using CLAMS suggests that SR1555 stimulated activity and increased energy expenditure in DIO mice.

**Induction of Thermogenesis by SR1555.** *UCP1* (uncoupling protein 1) is critical for heat generation, and the protein is selectively expressed in brown and beige adipose cells. Classic brown adipose tissue and inducible white adipocytes (beige cells) are capable of increasing energy expenditure through the uncoupling of oxidative metabolism from ATP (Wu et al., 2012, 2013). Also, previous physiologic studies have shown that diet can induce thermogenesis to maintain body weight (Rothwell and Stock, 1997). Therefore, we asked whether SR1555 induces thermogenic genes in adipose depots in DIO mice. Inguinal white adipose tissue and brown adipose tissue were collected from DIO mice. Induction of *UCP1* protein in inguinal white adipose tissue and overall adipose size were smaller in SR1555-treated inguinal white adipose



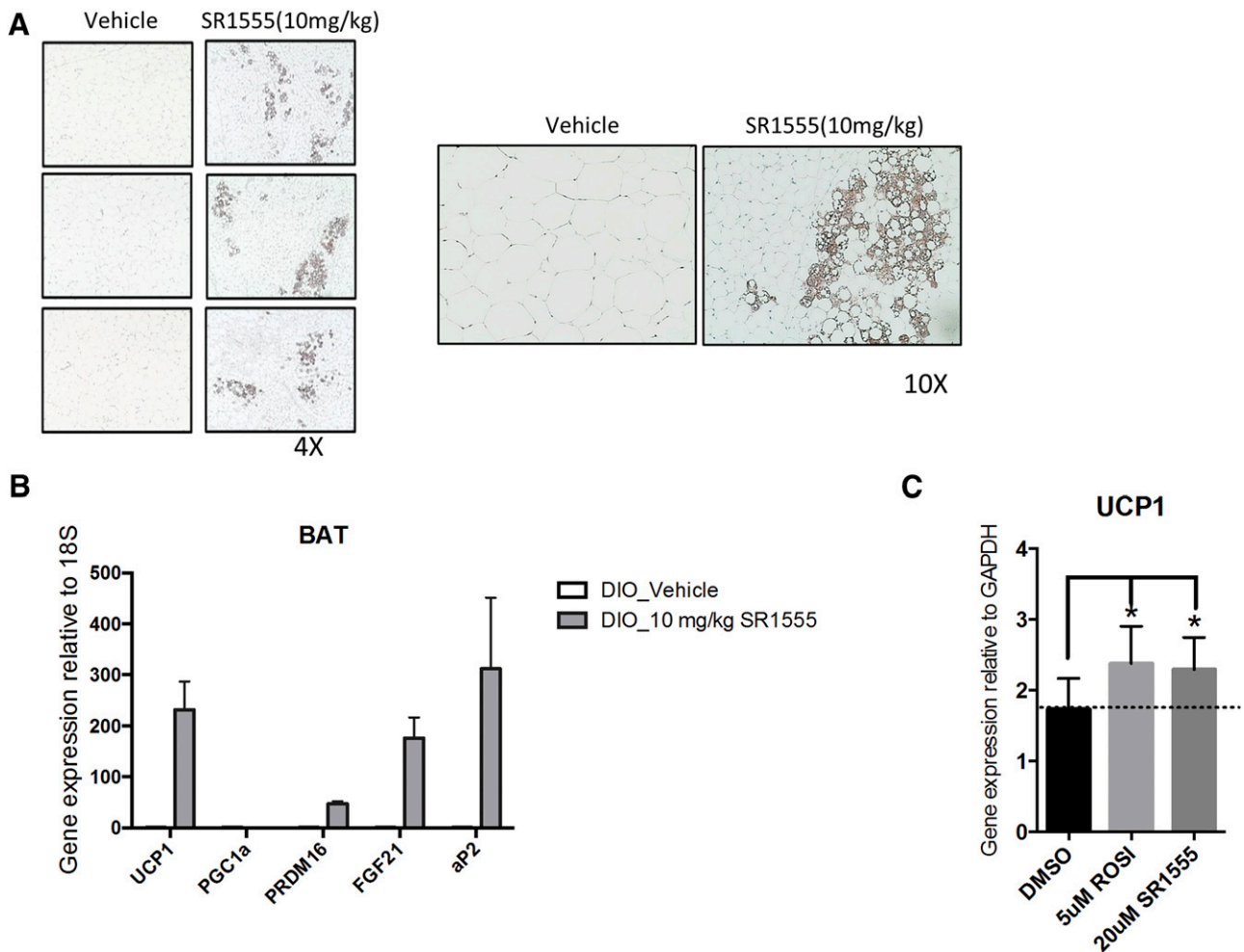
**Fig. 4.** In vivo measurement of energy expenditure and motor activity. (A) Total oxygen consumption (VO<sub>2</sub>), (B) total horizontal motor activity (XTOT), (C) total vertical motor activity (ZTOT), and (D) ambulatory activity count (XAMB). Mice were placed into the CLAMS for 5 days after one week (SR1555-treated obese mice). Values from all animals were pooled and averaged with an  $N = 5$  per group. Data were analyzed based on light and dark cycles. The light cycle occurred from 7:00 AM to 7:00 PM and the dark cycle occurred from 7:00 PM to 7:00 AM in the metabolic cages. Values are the mean  $\pm$  S.E.M. of 10 samples per group. \* $P < 0.05$ , \*\* $P < 0.01$  versus vehicle (Veh), by Student's unpaired  $t$  test.

tissue (Fig. 5A). Additionally, gene expression of *UCP1*, *PRDM16*, and *FGF21* was increased in brown adipose tissue from SR1555-treated mice compared with tissue from vehicle only-treated mice. The expression of the 18S gene was used as a control to determine the relative expression of thermogenic genes (Fig. 5B).

*FABP4* (aP2) gene expression was also increased by SR1555 treatment, and this phenotype was similar to that observed with genetic depletion of ROR $\gamma$  (Meissburger et al., 2011). SR1555 treatment induced *UCP1* gene expression in C3H10T1-2 cells (Fig. 5C), where the effect of SR1555 treatment was similar to that of thiazolidinedione treatment, which was the positive control for the assay (Ohno et al., 2012). Furthermore, SR1555 increased the mitochondrial membrane potential and mass in C2C12 cells (Fig. 6A). Taken together, we conclude that SR1555 induces the thermogenic gene program and alters mitochondrial potential in adipose and muscle cell lines.

**Fatty Acid Oxidation and Lipolysis.** The reduction of FFAs observed in obese mice treated with SR1555 could be the result of increased FFA storage, increased degradation of FFAs by  $\beta$ -oxidation, a decrease in lipolysis of triglycerides, or a combination of these events. The effect of SR1555 on fatty

acid oxidation was monitored in immortalized SVF cells. Differentiation was achieved in vitro by sequential application of adipogenic factors to preadipocytes over a period of several days. Following differentiation, adipocytes were characterized using the XF Analyzer from Seahorse Bioscience to measure four essential parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiration. Fatty acid oxidation in mitochondria is mainly controlled by *cpt1* (carnitine palmitoyl transferase I), which facilitates transport of long chain fatty acids into mitochondria. *cpt1* was unchanged in SR1555-treated SVF cells. However, oxygen consumption was increased at four independent time points following addition of a fatty acid mixture (oleic and linoleic acids), whereas no increase was observed in dimethylsulfoxide only-treated SVF cells (Fig. 6B). Next, we evaluated the activity of HSL in differentiated 3T3L1 cells following SR1555 treatment (Supplemental Fig. 6). Elevated plasma levels of FFAs are thought to play a major role in the pathogenesis of insulin resistance and type 2 diabetes by inhibiting glucose uptake and utilization by muscle and causing increased glucose output by the liver (Claus et al., 2005; Grousse et al., 2013). Here, we observed that *HSL* gene expression and phosphorylated HSL



**Fig. 5.** Improvement of thermogenic genes by SR1555 and *UCP1* induction. (A) Immunohistochemical examination of the presence of *UCP1*. Inguinal adipose tissues from vehicle-treated DIO mice or 10 mg/kg SR1555-treated DIO mice were stained brown for *UCP1* immunoreactivity. (B) mRNA expression of thermogenic genes in brown fat tissue (BAT) of mice fed with either vehicle-treated DIO or 10 mg/kg SR1555-treated DIO. (C) *UCP1* mRNA expression in adipogenic C3H10T1/2 cell line. C3H10T1/2 cells were induced adipogenesis with dexamethasone, insulin, and isobutylmethylxanthine for 2 days, and then maintained with insulin and each compound for 5 days. DMSO, dimethylsulfoxide; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; ROSI, Rosiglitazone.

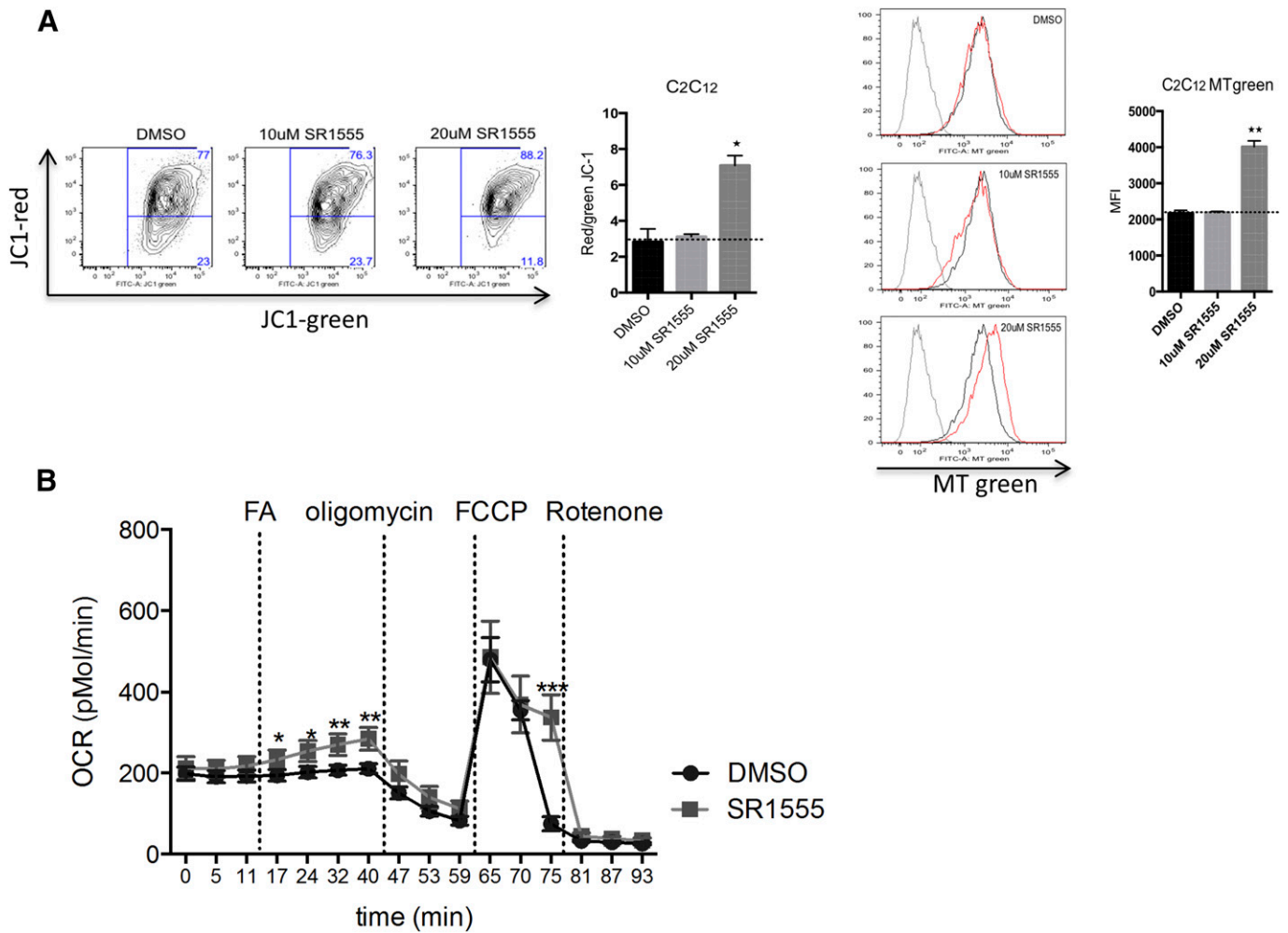
(S660) were reduced in fully differentiated 3T3L1 cells. Therefore, we suggest that decreasing phosphorylated HSL might drive the reduction of FFA release from white adipocytes.

## Discussion

The NR superfamily of ligand regulated transcription factors has proven to be a rich source of targets for the development of therapeutics for a wide range of human diseases. Within the NR1F subfamily of NRs, *ROR $\gamma$* T has garnered much attention due to its role in  $T_H17$  cells. However, its contribution to obesity and insulin resistance has been recently demonstrated (Meissburger et al., 2011; Takeda et al., 2014). The discovery that the potent LXR agonist T0901317 [*N*-(2,2,2-trifluoroethyl)-*N*-(4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl)-benzenesulfonamide] also functioned as an inverse agonist for *ROR $\alpha$*  and *ROR $\gamma$*  (Kumar et al., 2010) led to an explosion in medicinal chemistry efforts focused on the NR1F subfamily of receptors. This effort is exemplified by publications of the *ROR $\alpha$ / $\gamma$*  agonist SR1078 [*N*-(4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl)-4-(trifluoromethyl)benzamide]

(Wang et al., 2010), the selective *ROR $\alpha$*  inverse agonist SR3335 [*N*-(4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl)-2-thiophenesulfonamide] (Kumar et al., 2011), the dual *ROR $\alpha$ / $\gamma$*  inverse agonist SR1001 [*N*-(4-methyl-5-[[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]amino]sulfonyl]-2-thiazolylacetamide)] (Solt et al., 2011), and the selective *ROR $\gamma$*  inverse agonists SR2211 [1,1,1,3,3,3-hexafluoro-2-(4'-((4-(pyridin-4-ylmethyl)piperazin-1-yl)methyl)-[1,1'-biphenyl]-4-yl)propan-2-ol)] (Kumar et al., 2012) and SR1555 (Solt et al., 2012). Here, we demonstrate that the selective *ROR $\gamma$*  inverse agonist SR1555 recapitulates well the *ROR $\gamma$*  depletion phenotype particularly in terms of adiposity and metabolic parameters. Specifically, SR1555 treatment of obese and diabetic mice modulated adipocyte function, leading to a reduction in body weight exclusively as a result of reduced adiposity leading to improved insulin sensitivity and glucose disposal.

It is possible that SR1555-mediated improvement in metabolic phenotype involves the modulation of Treg development. Previously, it was shown that in cultured cells SR1555 repressed  $T_H17$  cell differentiation with concomitant expansion of inducible Tregs (Solt et al., 2012), and induction of Tregs has



**Fig. 6.** Fatty acid oxidation and modulation of mitochondrial respiration. (A) Functional mitochondria content in C2C12 cells was analyzed by flow cytometry using JC-1 and red/green JC-1 (the high-low mitochondrial membrane potential) mean fluorescent index (MFI) ratio (right,  $n = 3$  per condition). Mitochondria content was measured by flow cytometry analysis using Mito Tracker (MT) Green staining ( $n = 3$  per condition). (B) SVF cells were grown to confluence; incubated with dexamethasone, insulin, isobutylmethylxanthine, triiodothyronine (T3), and Rosiglitazone for 2 days to induce adipocyte differentiation; and then cultured with 20  $\mu$ M SR1555, insulin, and T3. Oxygen consumption rates (OCRs) were measured by a Seahorse XF-96 flux analyzer ( $n = 10$ , three different conditions). Values are the mean  $\pm$  S.E.M. of 10 samples per group. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus dimethylsulfoxide (DMSO), by Student's unpaired  $t$  test. FA; fatty acid (oleic and linoleic acids); FCCP, carbonylcyanide  $p$ -trifluoromethoxyphenylhydrazone.

been shown to be associated with metabolic disorders (Michalek et al., 2011; Bhat et al., 2014). Additionally, obese individuals with insulin resistance display a sharp reduction of Tregs (DeFuria et al., 2013), and depletion of Treg cells in mice using an anti-CD25 monoclonal antibody exacerbates insulin resistance (Eller et al., 2011). Previously, we had shown that SR1555 was capable of suppressing inflammatory cytokine production such as IL-6 and TNF- $\alpha$  in lipopolysaccharide-stimulated RAW264.7 cells (Chang et al., 2014). Therefore, it is possible that the anti-inflammatory actions of SR1555 are associated with its ability to improve adiposity and metabolic parameters.

Treatment of 3T3L1 cells with SR1555 resulted in inhibition of the enzyme HSL (EC 3.1.1.79). HSL is highly expressed in adipose tissue and is a major contributor to the formation of FFAs in fat. Release of these FFAs provides a source of energy for most tissues. HSL activity is the rate-limiting step in catecholamine-induced lipolysis, and the level of HSL expression is directly related to the lipolytic capacity of mature fat cells (Large et al., 1998). These initial findings

suggested that increased HSL activity may contribute to the increased plasma FFAs observed in obesity. Interestingly, partial inhibition of lipolysis in adipose tissue in HSL haploinsufficient mice (HSL $^{+/-}$ ) resulted in improved insulin sensitivity (Girousse et al., 2013). Analysis of the promoter region of *HSL* (LIPE) suggests it contains four putative RORE sequences (Supplemental Fig. 7, A and B). Thus, it is plausible that *HSL* expression and activity are regulated by ROR $\gamma$ . Previous chromatin immunoprecipitation sequencing data demonstrated that ROR $\gamma$  binds to the promoter of family members of *HSL*. It remains to be determined if SR1555-mediated repression of HSL activity is direct or indirect.

Furthermore, SR1555 treatment increased gene expression and protein levels of *UCP1*, altered mitochondrial potential, and increased the level of fatty acid oxidation in both brown adipocytes and beige cells, suggesting that ROR $\gamma$  regulates the oxidative phosphorylation process in mitochondria. In summary, the selective ROR $\gamma$  inverse agonist SR1555 may provide a useful starting point for the development of peripheral acting therapeutics for the treatment of diabetes and obesity.



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## Authorship Contributions

*Participated in research design:* Chang, Kamenecka, White, Griffin, Cameron.

*Conducted experiments:* Chang, Garcia-Ordenez, Kuruvilla, T. Khan, S. Khan, Lin, Unger.

*Contributed new reagents or analytic tools:* He, Kamenecka.

*Performed data analysis:* Chang, Unger, White, Griffin, Cameron.

*Wrote or contributed to the writing of the manuscript:* Chang, Corzo, Griffin, Cameron.

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**Address correspondence to:** Patrick R. Griffin, The Scripps Research Institute, Scripps, Florida, 130 Scripps Way, Jupiter, FL 33458. E-mail: pgriffin@scripps.edu