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Nur77 Regulates Lipolysis in Skeletal Muscle Cells

EVIDENCE FOR CROSS-TALK BETWEEN THE β-ADRENERGIC AND AN ORPHAN NUCLEAR HORMONE RECEPTOR PATHWAY*

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Megan A. Maxwel1‡, Mark E. Cleasby§, Angus Harding¶, Annika Stark¶, Gregory J. Cooney§, and George E. O. Muscat‡‖

From the Institute for Molecular Bioscience ‡Division of Molecular Genetics and Development and ¶Division of Molecular Cell Biology, University of Queensland, St. Lucia Queensland 4072 and §Diabetes and Obesity Research Program, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, New South Wales 2010 Australia

Skeletal muscle is a major mass peripheral tissue that accounts for ~40% of total body weight and 50% of energy expenditure and is a primary site of glucose disposal and fatty acid oxidation. Consequently, muscle has a significant role in insulin sensitivity, obesity, and the blood-lipid profile. Excessive caloric intake is sensed by the brain and induces β-adrenergic receptor (β-AR)- mediated adaptive thermogenesis. β-AR null mice develop severe obesity on a high fat diet. However, the target gene(s), target tissues(s), and molecular mechanism involved remain obscure. We observed that 30–60 min of β-AR agonist (isoprenaline) treatment of C2C12 skeletal muscle cells strikingly activated (>100-fold) the expression of the mRNA encoding the nuclear hormone receptor, Nur77. In contrast, the expression of other nuclear receptors that regulate lipid and carbohydrate metabolism was not induced. Stable transfection of Nur77-specific small interfering RNAs (siNur77) into skeletal muscle cells repressed endogenous Nur77 mRNA expression. Moreover, we observed attenuation of gene and protein expression associated with the regulation of energy expenditure and lipid homeostasis, for example AMP-activated protein kinase γ3, UCP3, CD36, adiponectin receptor 2, GLUT4, and caveolin-3. Attenuation of Nur77 expression resulted in decreased lipolysis. Finally, in concordance with the cell culture model, injection and electrotransfer of siNur77 into mouse tibialis cranialis muscle resulted in the repression of UCP3 mRNA expression. This study demonstrates regulatory cross-talk between the nuclear hormone receptor and β-AR signaling pathways. Moreover, it suggests Nur77 modulates the expression of genes that are key regulators of skeletal muscle lipid and energy homeostasis. In conclusion, we speculate that Nur77 agonists would stimulate lipolysis and increase energy expenditure in skeletal muscle and suggest selective activators of Nur77 may have therapeutic utility in the treatment of obesity.

Obesity and fat deposition are primarily controlled by food intake and energy expenditure. Obesity has been linked to the development of heart disease, cancer, and stroke, the top three causes of death in the United States, and has recently been recognized by the World Health Organization as one of the top 10 global health problems. Moreover, obesity leads to metabolic syndrome X, a disorder that includes elevated levels of triglycerides and low density lipoprotein (bad) cholesterol, low levels of high density lipoprotein (good) cholesterol, impaired fasting glucose, and hypertension (2). These are cardiovascular risk factors for diseases such as atherosclerosis and type II diabetes. More than 65% of United States adults are overweight, and >30% of adults (greater than 61 million people) are clinically obese (1, 2).

Diet, lifestyle, metabolism, and genetics are the most significant factors in controlling weight. Current obesity therapies perform modestly and are aimed at central appetite suppression and reduced fat absorption. Recent research suggests that therapeutic strategies which control satiety, reduce cellular energy stores, and increase adaptive thermogenesis and lipid catabolism provide new hope in the quest for novel anti-obesity drugs (1, 2).

Mechanisms exist that sense dietary excess and trigger increases in energy expenditure in an effort to preserve the fat mass set point. This process is responsive to cold and diet and results in the uncoupling of fuel oxidation from ATP synthesis, with the energy/proton gradient released in the form of heat rather than ATP production and is, thus, termed "adaptive thermogenesis."

Known components of the energy expenditure system include leptin, leptin receptor, α-melanocyte-stimulating hormone, melanocortin-4 receptor, and the β-adrenergic receptors (β-ARs).[1] However, many questions remain unanswered, including the hardwiring of the central neural circuits regulating energy balance, the efferent pathways, target tissues, target genes, and intracellular mechanisms involved (Refs. 3 and 4 and references therein).

The sympathetic nervous system (SNS) is an important efferent pathway that has been implicated in the control of adaptive thermogenesis. The evidence includes the following. Cold and diet induce increases in SNS activity, epinephrine stimulates energy expenditure, brown fat (a thermogenic tar-

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‖ A Principal Research Fellow of the National Health and Medical Research Council of Australia. To whom correspondence should be addressed. Tel.: 61-7-3346-2222; E-mail: g.muscat@imb.uq.edu.au.

[1] The abbreviations used are: β-AR, β-adrenergic receptor; siRNA, small interfering RNA; CAV3, caveolin 3; SREBP-1c, sterol regulatory element binding protein-1c; AMPK, AMP-activated protein kinase; LPL, lipoprotein lipase; GLUT4, glucose transporter 4; UCP, uncoupling protein; SNS, sympathetic nervous system; NR, nuclear hormone receptor; PPAR, peroxisome proliferator-activated receptor; Q-RT-PCR, quantitative real time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CPT-1, carnitine palmitoyl transferase 1; LXR, liver X receptor; ERR-1, estrogen-related receptor, alpha; ROR, RAR-related orphan receptor; COUP-TF, chicken ovalbumin upstream promoter.

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get tissue) is heavily innervated by the SNS, and thermogenic activity is dependent on an intact SNS. β-ARs and agonists transmit the thermogenic signals to peripheral tissues and mediate SNS activity (for review, see Refs. 3–5). Based on the above findings, a model has been proposed by Lowell and Bachman (3) that highlights the key aspects of adrenergic-induced thermogenesis: diet → brain → SNS → β-ARs → target tissue → thermogenesis.

The uncoupling proteins (UCP1–3) allow proton "leakage" across the inner mitochondrial membrane for the purpose of heat generation at the expense of coupled ATP production. The mechanism of adaptive thermogenesis in response to cold exposure has been elucidated and involves UCP1, which is highly expressed in brown fat. Knock-out experiments in rodents have demonstrated that UCP1 and brown fat mediate cold-induced thermogenesis; however, these animals are not obese and are resistant to diet-induced obesity (6). Knock-out mouse models of the UCP1 homologs, UCP2 and -3 (which are expressed in white adipose and skeletal muscle), have not provided clarification for a role in maintaining energy expenditure in that their responses to cold and high fat feeding are normal, and they show normal body weight and cold tolerance (for review, see Refs. 3 and references therein; Refs. 7–10). However, some resolution and understanding of the potential mechanisms controlling diet-induced thermogenesis has come from β-AR-less mice that are obese and completely lack both cold and diet-induced thermogenesis, indicating that β-AR play a role in both responses (5). However, identification of the target tissues relevant to diet-induced thermogenesis has remained elusive, and the possibilities include skeletal muscle, white adipose tissue, and liver, all of which are innervated by the SNS (3, 5).

Skeletal muscle is one of the most metabolically demanding major mass peripheral tissues that accounts for 50% of energy expenditure. Moreover, this lean tissue relies heavily on fatty acids as an energy source, accounts for 75% of glucose disposal, and is involved in cholesterol efflux. Consequently, muscle has a significant role in insulin sensitivity and the blood-lipid profile. These facts alone suggest that muscle has an important role in obesity and suggest muscle may have an important role in diet-induced modulation of energy expenditure.

The orphan nuclear hormone receptor NR4A subgroup is expressed in skeletal muscle, and intriguingly, NR4A1/Nur77 induction by β-AR agonists has been described in a single report (11). As mentioned above, the β-AR pathways have been implicated in responses to diet-induced thermogenesis. Moreover, skeletal muscle is rapidly emerging as a critical target tissue in the battle against obesity (12, 13), metabolic syndrome X (14), type II diabetes (15), and dyslipidemia (12–14,16). Nuclear hormone receptors (NRs) in skeletal muscle, for example LXR (17) and PPARα (18), -β/δ (14) and -γ (15), have been shown to be involved in enhancing insulin-stimulated glucose disposal rate, decreasing triglycerides, and increasing lipid catabolism, cholesterol efflux, and plasma high density lipoprotein C levels. Hence, understanding the link between the Nur77 orphan nuclear receptor, the β-adrenergic response, and energy expenditure (adaptive thermogenesis) has important implications for obesity.

This study focuses on the molecular regulation of gene expression involved in lipid and energy homeostasis in skeletal muscle cells by orphan nuclear receptors. We hypothesize that Nur77 (NR4A1) is involved in mediating β-AR signaling and that skeletal muscle is a critical target tissue in the regulation of energy expenditure.

Our investigation utilizes small interfering RNA (siRNA)-mediated knock-out of Nur77 in skeletal muscle cells to understand the regulatory role of a β-AR agonist-induced nuclear hormone receptor in this major mass peripheral tissue. 30–60 min β-AR agonist treatment of muscle cells strikingly activated (>100-fold) the expression of the mRNA encoding Nur77. In contrast, the expression of the other nuclear receptors was not induced. Furthermore, the study demonstrates that Nur77 is necessary for the expression of a subgroup of genes involved in energy balance and expenditure, including AMPKγ3 and UCP3. This is consistent with the observation that this orphan nuclear receptor is hypersensitive to adrenergic stimulation.

MATERIALS AND METHODS

Cell Culture—Mouse myogenic C2C12 cells were cultured in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated Serum Supreme (BioWhittaker, Edward Keller Pty Ltd., Hallam, Victoria, Australia)) in 6% CO_2. Myoblasts were differentiated into post-mitotic multinucleated myotubes by 5 days of serum withdrawal (*i.e.* cultured in Dulbecco's modified Eagle's medium supplemented with 2% horse serum). Cells were harvested at the indicated time points, usually 120 h (5 days) after mitogen withdrawal, unless indicated differently. African green monkey kidney COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. For the β-adrenergic agonist experiments, myotubes were treated with 100 nM isoprenaline hydrochloride (Calbiochem) or the vehicle control, ethanol, for the indicated time intervals.

Transient Transfections—Each well of a 24-well plate of COS-1 cells (~60% confluence) was transfected with a total of ~0.6 μg of DNA using the liposome-mediated transfection procedure as described previously (13). Cells were transfected using a DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate and Metafectene (Biontex Laboratories GmbH, Munich, Germany) liposome mixture in 1× HEPES-buffered saline (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na_2HPO_4, 11 mM dextrose (pH 7.1)). The DNA/DOTAP/Metafectene mixture was added to the cells in 0.6 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. 16 h later the culture medium was changed, and after a further 24 h the cells were harvested for the assay of luciferase activity. Values are expressed as relative light units ± S.D. and were derived from a minimum of two independent experiments composed of six replicates.

C2C12 Stable Transfection—Myogenic C2C12 cells cultured in growth medium were transfected with pSilencer 2.1-siNur77(DE) by the liposome-mediated procedure. The cells were then grown for another 24 h to allow cell recovery and neomycin resistance expression before G418 selection. After 10–14 days of selection with 500 μg/ml G418 (Invitrogen) in culture medium, the polyclonal pool (>50 G418-resistant colonies) of stable transfectants were cultured and maintained on 50 or 250 μg/ml G418 medium.

RNA Extraction and cDNA Synthesis—Total RNA was extracted from C2C12 cells and tibialis cranialis muscles (after grinding and homogenization of muscle tissue) using TRI-Reagent (Sigma-Aldrich) according to manufacturer's protocol. This was followed by DNase treatment with 2 units of Turbo DNase (Ambion, Austin, TX) for 1 h followed by heating to 75 °C for 10 min to inactivate the DNase. RNA for quantitative real time PCR was further purified using the RNeasy RNA extraction kit (Qiagen, Clifton Hill, Victoria, Australia) according to manufacturer's instructions. RNA was quantitated using UV spectrophotometry, and complementary DNA was synthesized from 3 μg of total RNA using an oligo$(dT)_{18}$ primer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Quantitative Real Time (Q-RT)-PCR—Target cDNA levels were analyzed by Q-RT-PCR in 25-μl reactions containing either 1× SYBR green (ABI, Warrington, UK) or Taqman PCR master mix (ABI, Branchburg, NJ), 200 nM each forward and reverse primers or 1× Assay-on-Demand Taqman primers (ABI, Foster City, CA), and the equivalent of 0.3 μl of cDNA. Using an ABI Prism 7000 sequence detection system, PCR was conducted over 45 cycles of 95 °C for 15 s and 60 °C for 1 min preceded by an initial 95 °C for 10 min to activate the Amplitaq Gold DNA polymerase. The relative level of expression or fold change and associated errors were calculated using the guidelines described by Bookout and Mangelsdorf (19) on the Nuclear Receptor Signaling Atlas website (NURSA) (www.nursa.org/index.cfm) in accord with the accepted Q-PCR standards for National Institutes of Health-supported NURSA research.

Primers—Primers used for Q-RT-PCR analysis of the mRNA populations have been described in detail (13), with the exception of primers designed for the detection of Nur77 using SYBR green (Nur77-F, TG-

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ATGTTCCCGCCTTTGC; Nur77-R, CAATGCGATTCTGCAGCTCTT). Assay-on-demand primer/probe sets for Nur77, AMPKγ1, and AMPKγ3 (Applied Biosystems) were also used for Taqman real time PCR.

Plasmids—POMC-tkLUC, pSG5-Nur77, and pSG5 have been described previously (20, 21). The pairs of oligonucleotides used for cloning the Nur77 siRNA targets into the pSilencer 2.1 and 3.1 vector were as follows: AB region, GATCCCGCTTCCAGCCGTCCCAGCTTTTCAAGAGAAAGCTGGGACGGCTGGAAGTTTTTTGGAAA and AGCTTTTTCCAAAAAACTTCCAGCCGTCCCAGCTTTCTCTTGAAAAGCTGGGACGGCTGGAAGCGG; DE region, GATCCCGTCCCTGGCTTCATTGAGCTTTCAAGAGAAGCTCAATGAAGCCAGGGATTTTTTGGAAA and AGCTTTTCCAAAAAATCCCTGGCTTCATTGAGCTTCTCTTGAAAGCTCAATGAAGCCAGGGACGG. These pairs of oligonucleotides were annealed and cloned into both the pSilencer 2.1 neo and pSilencer 3.1 neo expression vectors (Ambion, Austin, TX) as per the manufacturer's protocol.

In Vivo Electrotransfer into Mice—Plasmid vectors were propagated in ampicillin-selective media. DNA was extracted, purified using endotoxin-free maxi-prep kits (Qiagen Pty Ltd., Doncaster, Victoria, Australia), and re-suspended in sterile 0.9% saline. Anesthesia was induced in mice using 5% halothane in oxygen and maintained with 1–2% halothane in oxygen with administration of 5 mg/kg ketoprofen to provide post-operative analgesia. The hindlimbs of mice were shaved and prepared with a chlorhexidine-ethanol solution, and one tibialis cranialis muscle was injected in oblique fashion transcutaneously along their length with 30 μl of saline containing 0.5 mg/ml of each vector (pEGFP and pSilencer 2.1-siNur77(DE)) using a 29-gauge needle. The injection was immediately followed by the application of a pair of caliper electrodes across the injected leg connected to an ECM-830 electroporator device (BTX, Holliston, MA). Eight 20-ms pulses of 200 V/cm at a frequency of 1 Hz were administered as described previously (22). The other tibialis cranialis muscle was injected in the same fashion with the appropriate control vectors (pEGFP and pSilencer 2.1 negative) and electroporated using the same procedure. After recovery, animals were monitored daily for body weight and general condition. No adverse effects on the animals were noted during the experiment. One week after electrotransfer mice were killed, and the tibialis cranialis muscles were collected and immediately frozen in liquid nitrogen and stored at −70 °C for subsequent analysis.

Western Blotting—Cell lysates normalized for protein concentration and resolved on SDS-PAGE gels were transferred to polyvinylidene difluoride or nitrocellulose membranes using semidry transfer. The molecular mass of resolved proteins was determined by comparison with Precision Plus markers (Bio-Rad). The membranes were probed with anti-CPT1-m (CPT1, muscle) polyclonal antibody (Alpha Diagnostic International, Inc., San Antonio, TX), anti-GLUT4 polyclonal antibody (a gift of Prof. David James, Garvan Institute of Medical Research), and anti-CAV3 monoclonal antibody (BD Transduction Laboratories, San Jose, CA) followed by appropriate horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories Inc., San Francisco, CA). The horseradish peroxidase conjugate was detected using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent substrate, Pierce), and immunoreactivity was visualized by autoradiography.

Lipolysis Assay—Triglyceride hydrolysis (lipolysis) was assessed in C2C12 cells and the C2C12:siNur77 cells using the adipolysis assay kit (Chemicon Australia Pty Ltd., Boronia, Victoria, Australia) as per the manufacturer's instructions. Briefly, C2C12 cells and C2C12-siNur77 cells were differentiated for 5 days, the culture media was removed, and the monolayer was washed twice with wash solution (Hanks' balanced salt solution). 250 μl of incubation solution (Hanks' balanced salt solution + 2% bovine serum albumin) was added to the wells in triplicate, and cells were incubated for 1, 3, and 6 h. At each time point the incubation solution was removed and stored at −20 °C until analyzed. To measure lipolysis, 200 μl of free glycerol assay reagent was added to 25 μl of culture supernatants and glycerol standards and incubated for 15 min, and the absorbance was read at 540 nm. A standard curve constructed from the glycerol standards was used to calculate the concentration of glycerol in the culture supernatants.

RESULTS

Nur77 mRNA Is Expressed in Skeletal Muscle Cells and Induced during Myogenesis—To elucidate the functional role of Nur77 in skeletal muscle, we initially investigated the expression of Nur77 mRNA relative to GAPDH in the mouse C2C12 myoblast cell line. Proliferating C2C12 myoblasts can be induced to biochemically and morphologically differentiate into

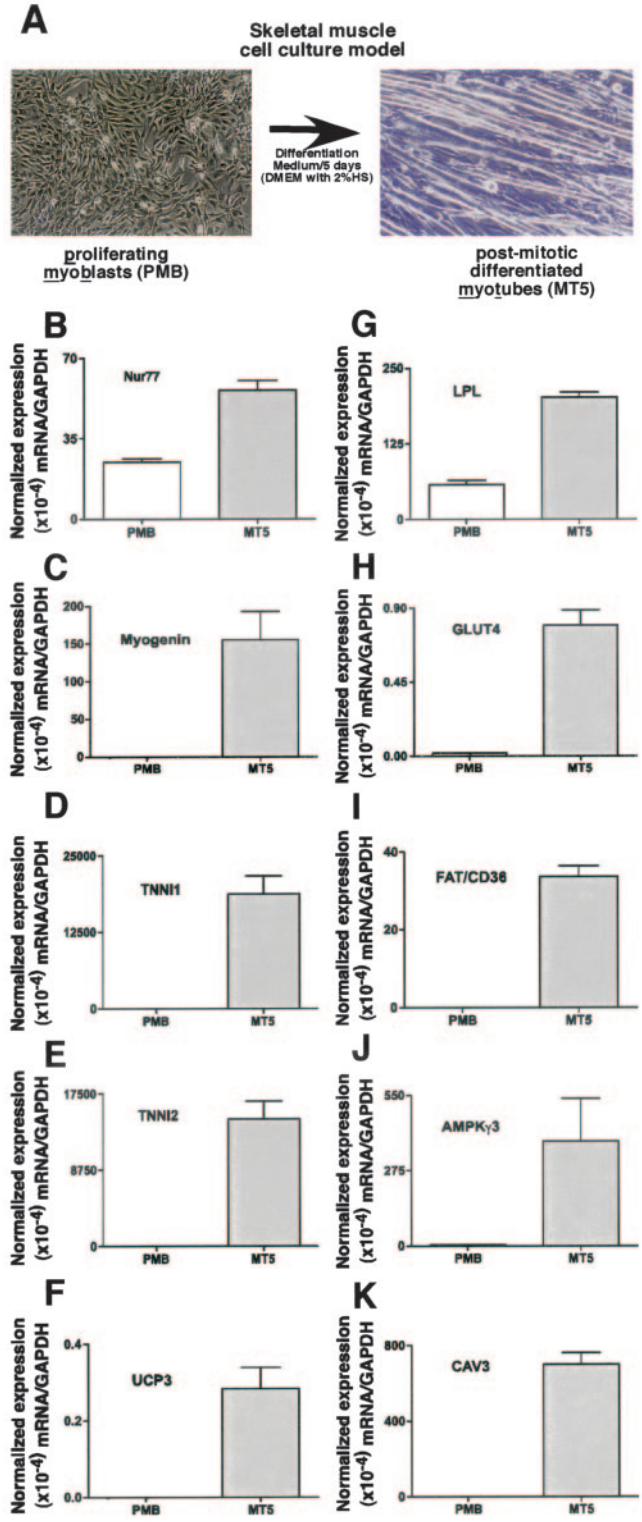

FIG. 1. **Nur77 mRNA is expressed and induced during myogenesis in skeletal muscle cells.** *A*, schematic illustration of the cell culture model. Proliferating myoblasts were differentiated into post-mitotic multinucleated myotubes by 5 days of serum withdrawal. *B–K*, Q-RT-PCR analysis of mRNA expression during skeletal myogenesis. RNA extracted from C2C12 PMB and MT5 was reverse-transcribed to cDNA and analyzed by Q-RT-PCR for each target gene. Results were normalized to GAPDH and presented as number of target transcripts per GAPDH transcript ±S.D. *B*, Nur77 mRNA levels; *C*, myogenin mRNA levels; *D*, troponin I type 1 (slow) (TNNI1) mRNA levels; *E*, troponin I type 2 (fast) TNNI2 mRNA levels; *F*, UCP3 mRNA levels; *G*, LPL mRNA levels; *H*, GLUT4 mRNA levels; *I*, fatty acid translocase (*FAT*)/CD36 mRNA levels; *J*, AMPKγ3 mRNA levels; *K*, CAV3 mRNA levels.

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TABLE I
Key target genes in this study

ABCA1 and ABCG8	ATP binding cassette. Transporters that transfer cholesterol to the high density lipoprotein acceptors, *i.e.* reverse cholesterol efflux.
ACS4	Acyl-CoA synthetase 4. Enhances the uptake of fatty acids by catalyzing their activation to acyl-CoA esters for subsequent use in catabolic fatty acid oxidation pathways.
ADRP/adipophilin	Adipocyte differentiation-related protein. Involved in lipid storage.
AdipoR1 and -R2	Adiponectin Receptors 1 and 2. Cell surface membrane receptors for adiponectin that regulate glucose uptake and fatty acid oxidation (38).
AMPK	5′-AMP-activated protein kinase. A fuel-sensing enzyme that responds to cellular stress by regulating carbohydrate and fat metabolism (60).
ApoE	Apolipoprotein E. Facilitates cholesterol and lipid efflux.
CAV3	Caveolin 3. Muscle-specific caveolin involved in membrane protein anchoring, caveolae, t-tubules formation, and lipid trafficking.
CPT-1	Carnitine palmitoyl transferase 1. Transfers the long chain fatty acyl group from coenzyme A to carnitine, the initial reaction of mitochondrial import of long chain fatty acids and their subsequent oxidation.
FAS	Fatty acid synthase. Involved in *de novo* fatty acid production.
FAT/fatty acid translocase and FABP3	Fatty acid translocase, and fatty acid binding protein. Facilitate uptake of long chain fatty acids (LCFAs) and low density lipoproteins.
GLUT4 and -5	Glucose transporters. GLUT4 facilitates glucose uptake in response to insulin stimulation. GLUT5 catalyzes uptake of fructose.
Glycogenin/GYG1	Initiates the synthesis of glycogen, the principal storage form of glucose in skeletal muscle.
LPL	Lipoprotein lipase. Hydrolysis of lipoprotein triglycerides into free fatty acids and responsible for the uptake of free fatty acids.
PDK2 and -4	Pyruvate dehydrogenase kinases. Inhibits the pyruvate dehydrogenase complex, thereby controlling glucose oxidation and maintaining pyruvate for gluconeogenesis.
SCD1 and -2	Stearoyl CoA desaturase 1 and 2. Enzymes associated with adiposity, *i.e.* storage and esterification of cholesterol and responsible for the *cis* saturation of stearoyl and palmitoyl-CoA, converting them to oleate and palmitoleate, which are the monounsaturated fatty acids of triglycerides.
SREBP-1c	Sterol regulatory element binding protein 1c. The hierarchical transcriptional activator of lipogenesis.
UCP1, -2, and -3	Uncoupling proteins. Mitochondrial proteins that uncouple metabolic fuel oxidation from ATP-synthesis, regulating energy expenditure.

post-mitotic multinucleated myotubes by serum withdrawal in culture over a 48–96-h period (Fig. 1*A*).

We utilized the ABI Taqman Nur77 primer set and also used the GenBank™ sequences of Nur77 to design specific primers for the SYBR green mediated amplification of mouse Nur77 by quantitative real time PCR using total RNA isolated from proliferating and differentiated C2C12 cells. Total RNA was isolated from proliferating myoblasts and post-mitotic myotubes after 5 days (MT5) of serum withdrawal and converted to cDNA, and the expression levels of various genes was then examined by real time PCR.

We observed that Nur77 is weakly expressed in proliferating myoblasts; however, the transcript is induced ~4 fold relative to GAPDH mRNA as the cells exit the cell cycle and fuse to form differentiated multinucleated myotubes that have acquired a muscle-specific phenotype (Fig. 1*B*). Concomitant with this increase in Nur77 mRNA was the striking induction of the slow (type I, TNNI1) and fast (type II, TNNI2) isoforms of the contractile protein, troponin I, relative to GAPDH mRNA (Fig. 1, *D* and *E*). Moreover, we observed the significant induction of myogenin mRNA that encodes the hierarchical bHLH regulator (Fig. 1*C*). These data confirmed that the cells had terminally differentiated. Furthermore, we observed the induction of the mRNAs encoding UCP3 (Fig. 1*F*), LPL (Fig. 1*G*), the GLUT4 transporter (Fig. 1*H*), the fatty acid translocase, CD36 (Fig. 1*I*), the type II/glycolytic muscle specific AMPKγ3 (Fig. 1*J*), and caveolin 3 (CAV3, Fig. 1*K*). These are examples of mRNAs encoding proteins involved in the regulation of energy expenditure and lipid and carbohydrate metabolism (as described in Table I). In summary, these data demonstrate that the mRNA transcript encoding Nur77 was expressed in C2C12 cells in a differentiation-dependent manner, concomitant with the acquisition of a contractile and metabolic phenotype.

β-Adrenergic Agonist Treatment Transiently Induces Nur77 mRNA Expression in Skeletal Muscle Cells—We utilized the C2C12 cell culture model to examine whether regulatory crosstalk occurred between the β-adrenergic and nuclear hormone receptor signaling pathways in skeletal muscle. There has been

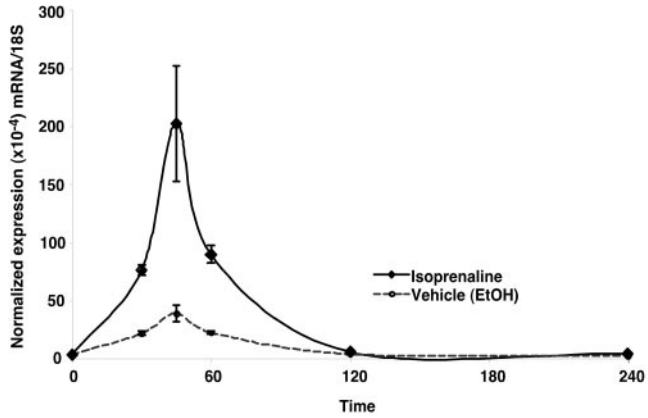

FIG. 2. ***β*-Adrenergic agonist (isoprenaline) treatment transiently induces Nur77 expression in skeletal muscle cells.** C2C12 MT5 cells were treated with vehicle (ethanol) or isoprenaline for 30, 45, 60, 180, and 240 min. Levels of Nur77 mRNA were quantitated by Q-RT-PCR. Expression was normalized and presented as the number of target transcripts per 18 S transcript ± S.D.

a single report describing the induction of Nur77 by adrenergic agonists in muscle (11), and a second manuscript reported that epinephrine induced Nur77 in the brain (23); however, these single observations have not been reproduced in the literature.

Therefore, to investigate and substantiate these conclusions, we treated differentiated post-mitotic skeletal muscle myotubes with the β-adrenergic agonist, isoprenaline (and the vehicle, ethanol) for 30, 45, 60, 120, and 240 min and subsequently isolated total RNA. Q-RT-PCR was utilized to examine the expression of mRNAs encoding Nur77 and a number of other nuclear hormone receptors that regulate lipid homeostasis. Interestingly, we observed that the mRNA encoding Nur77 was dramatically induced (>100-fold) after 30–60 min of incubation with isoprenaline (Fig. 2). Activation of expression was transient and declined after 60 min of treatment. There was a slight induction of Nur77 with ethanol alone, a response that has been noted in another report (24), and this is not surprising

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TABLE II
Relative expression of nuclear hormone receptors after vehicle and isoprenaline treatment, presented as the number of target transcripts per 18 S transcript

	Time				
	0[a]	30	45	60	120
			min		
Isoprenaline treated C2C12 myotubes[b]					
ERR-1	94.674 ± 0.504	137.654 ± 0.180	163.321 ± 1.056	121.789 ± 1.205	119.835 ± 0.268
PPARα	2.025 ± 0.012	2.735 ± 0.032	4.964 ± 0.017	2.171 ± 0.024	1.238 ± 0.007
PPARβ/δ	120.668 ± 0.975	176.261 ± 0.900	211.069 ± 1.238	203.408 ± 0.982	123.917 ± 0.034
PPARγ	15.400 ± 0.062	18.230 ± 0.042	30.659 ± 0.086	21.679 ± 0.025	16.129 ± 0.128
LXRα	3.895 ± 0.004	4.828 ± 0.001	7.336 ± 0.025	3.486 ± 0.014	3.283 ± 0.005
LXRβ	455.583 ± 2.470	673.205 ± 2.721	866.007 ± 3.611	538.040 ± 1.200	562.185 ± 6.576
RORα	1.432 ± 0.015	1.834 ± 0.006	6.154 ± 0.019	5.296 ± 0.021	0.850 ± 0.003
Rev-erbα	68.194 ± 0.118	79.428 ± 0.142	94.020 ± 0.539	51.562 ± 0.235	50.152 ± 0.570
RVR (Rev-erbβ)	7.020 ± 0.022	9.612 ± 0.026	27.824 ± 0.047	32.784 ± 0.087	4.107 ± 0.021
COUP-TFI	316.977 ± 2.650	427.042 ± 1.443	632.491 ± 3.361	437.024 ± 0.574	160.330 ± 0.626
COUP-TFII	170.651 ± 1.242	264.704 ± 0.739	455.583 ± 1.222	259.856 ± 0.997	114.423 ± 0.211
Vehicle (ethanol)-treated C2C12 myotubes[b]					
ERR-1	94.674 ± 0.504	191.107 ± 0.260	160.330 ± 0.893	202.471 ± 0.321	130.530 ± 0.277
PPARα	2.025 ± 0.012	4.262 ± 0.010	4.392 ± 0.019	3.000 ± 0.033	2.878 ± 0.020
PPARβ/δ	120.668 ± 0.975	213.521 ± 1.257	204.823 ± 0.260	262.269 ± 1.322	120.112 ± 0.324
PPARγ	15.400 ± 0.062	26.567 ± 0.095	28.212 ± 0.111	26.384 ± 0.104	42.466 ± 3.091
LXRα	3.895 ± 0.004	6.416 ± 0.061	7.037 ± 0.027	7.053 ± 0.009	3.626 ± 0.007
LXRβ	455.583 ± 2.470	956.465 ± 3.389	766.195 ± 6.138	876.070 ± 1.006	612.360 ± 2.709
RORα	1.432 ± 0.015	3.758 ± 0.021	4.292 ± 0.020	2.232 ± 0.018	1.872 ± 0.022
Rev-erbα	68.194 ± 0.118	115.486 ± 0.112	101.939 ± 0.267	96.887 ± 0.064	64.218 ± 0.363
RVR (Rev-erbβ)	7.020 ± 0.022	14.469 ± 0.068	13.345 ± 0.045	10.616 ± 0.017	8.121 ± 0.015
COUP-TFI	316.977 ± 2.650	682.603 ± 1.155	623.784 ± 4.209	403.074 ± 0.677	238.564 ± 2.291
COUP-TFII	170.651 ± 1.242	415.365 ± 1.099y	376.952 ± 1.937	246.977 ± 0.263	239.116 ± 0.665

[a] Untreated control sample.
[b] C2C12 cells were grown to confluence, differentiated for 72 h by serum withdrawal, then treated for the specified period. Values are expressed as mean ± S.D. of the expression relative to 18 S ($2^{(-\Delta Ct)} \times 10^{-6}$).

given that Nur77 has been implicated as a stress-response gene (25). Furthermore, we examined the expression of many other nuclear hormone receptors that have been demonstrated to regulate lipid and carbohydrate metabolism, including ERR-1, PPARα, -β/δ, and -γ, LXRα and -β, Rev-erbα, RVR, RORα, and COUP-TFI and II. In contrast to the striking induction of Nur77, the expression of the mRNAs encoding the NRs above did not respond to β-adrenergic agonist treatment (Table II).

In summary, these data clearly demonstrate that β-adrenergic agonists selectively and dramatically activated the mRNA encoding Nur77 (and *not* other nuclear receptors). This suggests that β-adrenergic agonists and Nur77 may have a unique regulatory footprint in skeletal muscle cells and demonstrates cross-talk between the β-adrenergic and an orphan nuclear hormone receptor pathway.

Nur77-siRNA Expression Attenuates Nur77-mediated Gene Expression and Represses Endogenous Levels of Nur77 mRNA—To understand the biological role of Nur77 in skeletal muscle lipid and energy homeostasis and to identify the metabolic target genes of this orphan receptor in muscle cells, we proceeded to examine the effect of ablation. This would provide a tool for elucidating the consequences of β-AR-mediated induction of Nur77. Furthermore, the analysis of the C2C12-siNur77 cell line would ascertain the extent of cross-talk between the β-adrenergic and Nur77-signaling pathways in the context of target genes and metabolic pathways.

We utilized RNA interference to achieve targeted silencing of Nur77 using double-stranded siRNA expression as the triggering agent. Two siRNA target sequences were chosen using the siRNA target finder on the Ambion web site (www.ambion.com/techlib/misc/siRNA_finder.html). The selected siRNAs (Fig. 3*A*) were cloned into the Silencer 2.1 and 3.1 plasmid expression vectors, driven, respectively, by the U6 and H1 RNA polymerase III promoters. The efficacy of the cloned siRNAs and their ability to attenuate trans-activation of the native Nur77-dependent heterologous reporter gene (Fig. 3*B*) in the presence of exogenous and overexpressed Nur77 was analyzed. This demonstrated that the Silencer 2.1 plasmid driving the siRNA targeting the C-terminal DE region (encoding the putative ligand binding domain of Nur77) most successfully attenuated Nur77-dependent gene expression.

We stably transfected C2C12 cells with the Silencer 2.1-siNur77(DE) vector and isolated a polyclonal pool of G418-resistant colonies (comprised of >50 G418 resistant colonies and, henceforth, denoted as C2C12-siNur77). Q-RT-PCR demonstrated the C2C12-siNur77 cells expressed reduced levels (~10-fold) of the Nur77 mRNA transcript relative to the native C2C12 cells (Fig. 3*C*). In conclusion, the C2C12-siNur77 cell line displayed attenuated Nur77 mRNA expression.

Exogenous Nur77-siRNA Represses the Expression of the mRNAs Encoding Genes That Regulate Lipid and Energy Homeostasis—The C2C12 *in vitro* cell culture system has been extensively used to investigate the regulation of lipid homeostasis (12, 13, 17, 26–30). Moreover, the physiological validation of the cell culture model with respect to the control of lipid metabolism by LXR and PPARs in the mouse (14, 17, 18, 31, 32) corroborates the utility of this model system.

Hence, we proceeded to examine the effect of attenuating Nur77 mRNA expression on the expression of genes involved in lipid and energy homeostasis (see Table I). We compared expression in the siNur77-expressing stable cell line relative to the wild type C2C12 cell line. Specifically, both cell lines were induced to differentiate into post-mitotic multinucleated myotubes by mitogen withdrawal in culture over a 5-day period and isolated total RNA from the native and siNur77-expressing cell line for Q-RT-PCR analysis.

Initially, we examined the expression of markers indicative of differentiation to ascertain whether the siNur77 cell line retained the potential to morphologically and biochemically differentiate. Over a 5-day period the C2C12-siNur77 cell line

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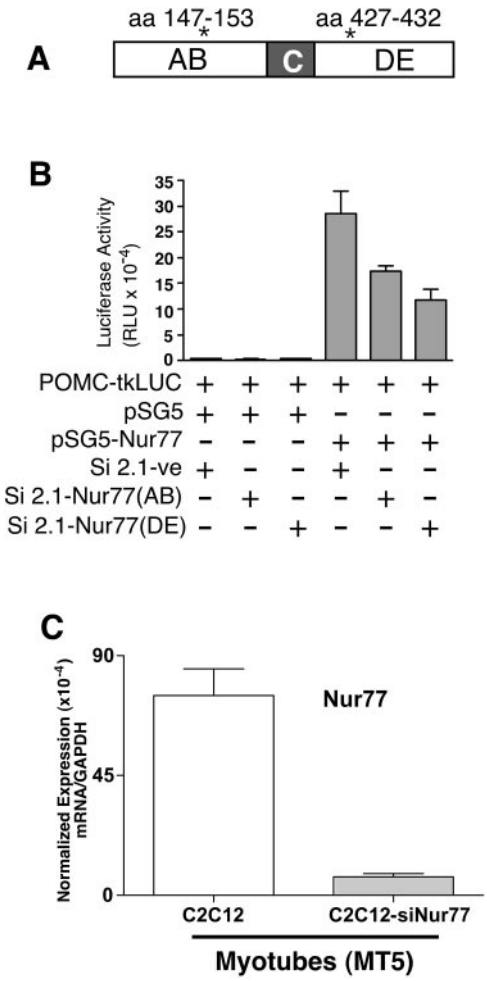

FIG. 3. **Nur77 siRNA attenuates Nur77-dependent trans-activation and represses the endogenous levels of Nur77 mRNA expression.** *A*, diagrammatic representation of Nur77 depicting the location of the N- and C-terminal siRNAs. *B*, pSG5-Nur77 and either pSilencer 2.1 negative or pSilencer 2.1-Nur77(DE) were co-transfected with POMCx5-TKLuc into COS-1 cells. Mean luciferase activity is expressed as relative light units (*RLU*) ± S.D. ($n = 6$). *C*, Q-RT-PCR analyses of Nur77 mRNA levels in native C2C12 and C2C12-siNur77 myotubes, with expression normalized and presented as the number of target transcripts per GAPDH transcript ± S.D. *aa*, amino acid.

retained the potential to morphologically differentiate. Moreover, the induction and activation of myogenin (Fig. 4*A*), troponin I slow (Fig. 4*B*, TNNI1), and troponin I fast (Fig. 4*C*, *TNNI2*) mRNA expression after serum withdrawal demonstrated that the cell line biochemically differentiated. Furthermore, we examined the relative expression of myogenin and TNNI1 and -2 in wild type C2C12 *versus* C2C12-siNur77 myotubes after 5 days of serum withdrawal. We observed that myogenin mRNA expression (Fig. 4*D*) was increased ~2-fold, whereas TNNI1 (Fig. 4*E*) and TNNI2 (Fig. 4*F*) mRNA expression were down-regulated ~3-fold.

For the sake of brevity we will only show the graphical data for a number of genes; the complete list of genes examined and their relative expression are presented in Table III. In the context of lipid and fatty acid absorption we observed significant (~8-fold) repression of the mRNA encoding fatty acid translocase (*FAT/CD36*, Fig. 5*A*) relative to the changes observed in the biochemical markers of differentiation. Moreover, the noteworthy repression of CD36 was highlighted by the minor changes (<2.5-fold) observed for fatty acid-binding protein 3 (FABP-3) and 4 (FABP-4) in the siNur77-expressing cell line, respectively (see Table III), similar to the minor changes observed in the biochemical markers of differentiation. These negligible alterations in the siNur77-expressing cell line correlated with the subtle up and down variations observed in the biochemical markers of differentiation, in contrast to the dramatic repression of CD36 mRNA expression.

The expression of the mRNA encoding the glucose transporter, GLUT4, was repressed ~10-fold (Fig. 5*B*) in the siNur77-expressing cell line. In contrast, the expression of the mRNA encoding the fructose transporter, GLUT5, was unchanged by Nur77 attenuation (Fig. 5*C*, Table III).

In the context of the regulation of energy expenditure and energy balance, we examined the expression of several genes (see Tables I and III). The expression of a small subgroup of genes emerged as being clearly dependent on Nur77 expression. For example, the mRNAs encoding UCP3 and AMPKγ3 (preferentially expressed in glycolytic fast muscle fibers) were significantly repressed (~6–20-fold; Fig. 5, *D* and *H*) in the siNur77-expressing cell line relative to the ~2-fold changes observed in the markers of differentiation (Fig. 4, *D–F*, Table III). Consistent with the effects on this subgroup of genes, we observed that adiponectin receptor 2 (Fig. 5*F*) (but not adiponectin receptor 1; Fig. 5*G*) was repressed ~4-fold). This receptor for the anti-diabetic adipokine Acrp30/adiponectin has been reported to activate AMPK and fatty acid oxidation.

Interestingly, the mRNA encoding the hierarchical regulator of lipogenesis, sterol regulatory element binding protein-1c (SREBP-1c), was increased ~4-fold; this is consistent with abrogation of AMPKγ3 mRNA expression, which in animals leads to excessive triglyceride accumulation (33). Surprisingly, the lipogenic downstream target genes including stearoyl-CoA desaturase 1 and 2 and fatty acid synthase (see Table III) were not affected in the siNur77-expressing cell line. The expression of the transcripts encoding LPL, muscle type carnitine palmitoyltransferase-1, medium chain acyl-CoA dehydrogenase, and acyl-CoA synthetase-4 (ACS4), which are involved in lipid catabolism and preferential fuel utilization, were not affected in the siNur77-expressing cell line (see Tables I and III).

Additionally, we did not observe any changes in the expression of mRNAs encoding ATP binding cassette A1, ATP binding cassette A8/G1), apolipoprotein E, adipophilin, and glycogenin-1 (see Tables I and III), involved in cholesterol homeostasis and lipid and glucose storage in the cells expressing the siNur77. However, we observed that the mRNA encoding caveolin-3, which has been implicated in lipid transport and storage (34, 35), was significantly suppressed (~13-fold, Fig. 5*J*) in the siNur77 cell line.

Furthermore, we examined the expression of many other nuclear hormone receptors that have been demonstrated to regulate lipid and carbohydrate metabolism, including ERR-1, PPARα, -β/δ, and -γ, LXRα and -β, Rev-erbα, RVR (Rev-erbβ), RORα, and COUP-TFI and -II and found that siNur77 expression did not affect the expression of the mRNAs encoding the other orphan NRs.

We utilized Western analysis to confirm that some of the critical changes in mRNA expression translated to changes in protein expression. For example, we examined the expression of caveolin 3 and GLUT4 expression in the native C2C12 and the siNur77-expressing cells because the mRNAs encoding these proteins were significantly repressed in the C2C12: siNur77 cell line. As a control we also examined the protein expression of CPT-1 because the mRNA encoding this enzyme was not affected by attenuation of Nur77 expression. Consistent with the mRNA expression profiling, we observed significant suppression of caveolin 3 and GLUT4 protein expression in the siNur77-expressing cells (Fig. 6, *A* and *B*), whereas CPT-1 protein expression remained unchanged (Fig. 6*C*).

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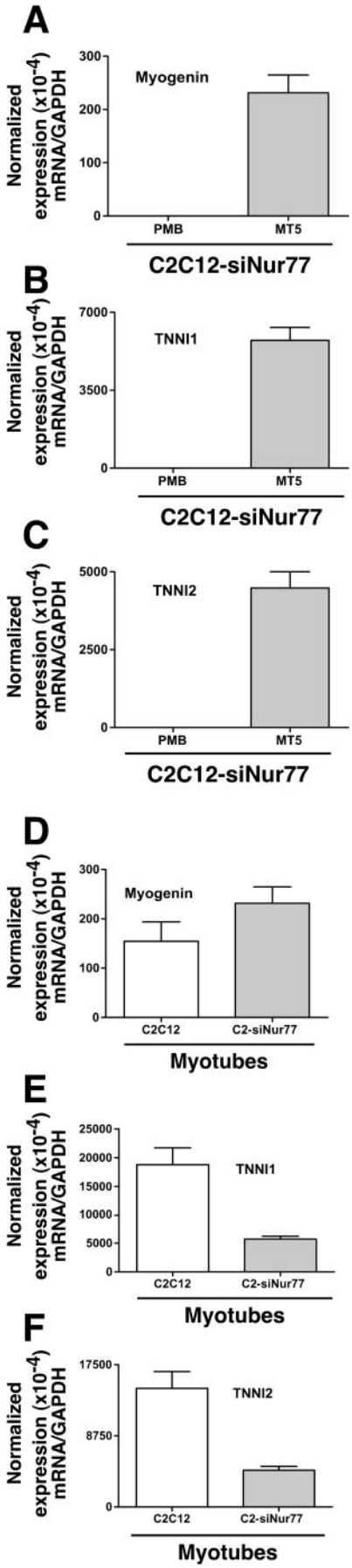

FIG. 4. **The C2C12-siNur77 cell line acquires biochemical markers of skeletal muscle.** *A*, *B*, and *C*, Q-RT-PCR analyses of myogenin and troponin I type I (slow) and II (fast) mRNA levels, respectively, in the C2C12-siNur77 cell line. *D*, *E*, and *F*, Q-RT-PCR analyses of myogenin and troponin I type I (slow) and II (fast) mRNA levels, respectively, in the native C2C12 and the C2C12-siNur77 cell line. Expression was normalized and presented as number of target transcripts per GAPDH transcript ±S.D. *PMB*, proliferating myoblasts; *MT5*, post-mitotic myotubes after 5 days.

In summary, the expression of a subgroup of genes involved in glucose absorption, lipid homeostasis, and the regulation of energy balance including GLUT4, CD36, CAV3, AMPKγ3, adiponectin receptor 2, and UCP3 emerged as clearly dependent on Nur77 expression. The Nur77-specific dependence of this subgroup of metabolic genes was underscored by the observation that related genes, for example GLUT5, UCP2, AMPKγ1, and adiponectin receptor 1, were refractory to a reduction in Nur77 expression.

Lipolysis Is Reduced in the siNur77-expressing Cells—Our study demonstrated that that a subgroup of genes that include adiponectin receptor 2, UCP3, and AMPKγ3 are repressed in the C2C12:siNur77 cell line. Studies in a variety of animal models suggest that suppression of these genes would lead to a reduction in fatty acid oxidation, reduced lipid utilization, and lipolysis. For example, muscle-specific overexpression of UCP3 leads to preferential lipid utilization (36, 37), adiponectin receptor 2 activation leads to increased β-oxidation (38), and AMPKγ3 null mice exhibit triglyceride accumulation (33).

To test this hypothesis in our cell culture system we examined lipolysis (triglyceride hydrolysis) in the native C2C12 cells and in the siNur77-expressing cells. Lipolysis/triglyceride breakdown was measured by examining glycerol release into the cell culture supernatant after 1-, 3-, and 6-h incubations. We utilized the Chemicon adipolysis kit to measure lipolysis and observed that glycerol release was significantly reduced from the C2:siNur77 cell line relative to the native C2C12 cell line (Fig. 7). This demonstrates that attenuation of Nur77 expression results in reduced lipolysis in skeletal muscle cells.

Expression of Nur77 siRNA in Mouse Tibialis Muscle Leads to Repression of UCP3 mRNA Expression—To examine whether the effects and changes observed in the skeletal muscle cell culture model were reflected in an *in vivo* animal model, we injected and electroporated mouse tibialis muscle with the Nur77 siRNA expression vector. Specifically, the right hindlimb tibialis cranialis muscle of three mice was injected in oblique fashion transcutaneously with the pSilencer 2.1 siNur77 expression using a 29-gauge needle followed by electrotransfer as described (22). The left hindlimb tibialis cranialis muscle was injected with the pSilencer 2.1 control vector and electroporated using the same procedure. One week after electrotransfer mice were sacrificed, the tibialis cranialis muscles were collected, and RNA was harvested for subsequent analysis. Expression of pSilencer 2.1-siNur77 produced an ~2-fold repression in Nur77 mRNA expression in the right tibialis cranialis muscle relative to the left muscle injected with the control silencer vector only (Fig. 8*A*). In agreement with the cell culture model, we observed a repression in UCP3 mRNA expression (Fig. 8*B*). This data confirmed that a key change reported in the cell culture model, *viz.* a down-regulation of UCP3 expression, was confirmed in mouse skeletal muscle.

DISCUSSION

Genetic, molecular, and biochemical studies have clearly demonstrated that β-adrenergic agonists and β-ARs are involved in the regulation of cold- and diet-induced thermogenesis and induce increases in energy expenditure (3–5). However, the target tissues, UCP1-independent mechanisms, and molecular pathways involved in diet-induced thermogenesis have not been resolved and remain a controversial area. One favorable

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TABLE III

Relative mRNA expression and -fold change for genes involved in metabolism from native C2C12 and C2C12-siNur77 cells

MCAD, medium chain acyl-CoA dehydrogenase; ADRP, adipocyte differentiation-related protein; FAS, fatty acid synthase; FABP, fatty acid-binding protein; PDK, pyruvate dehydrogenase kinase; SCD, stearoyl-CoA desaturase; ACS4, acyl-CoA synthetase 4; ABC, ATP binding cassette; PGC-1, peroxisome proliferator-activated receptor gamma, coactivator 1; GYG1, glycogenin.

Gene	Relative expression C2C12[a]	Relative expression C2C12-siNur77[a]	-Fold change[b]
Nuclear receptors			
Nur77	75.12 ± 9.87	7.07 ± 1.36	↓ 10.63 ± 2.47
ERR-1	21.62 ± 2.76	15.75 ± 1.70	↓ 1.37 ± 0.23
PPARα	0.58 ± 0.19	0.37 ± 0.13	↓ 1.56 ± 0.76
RVR (Rev-erbβ)	5.40 ± 1.33	8.01 ± 2.16	↑ 1.48 ± 0.54
Rev-erbα	8.21 ± 1.42	7.22 ± 2.18	↓ 1.14 ± 0.40
COUP-TFI	65.54 ± 5.81	130.18 ± 10.03	↑ 1.99 ± 0.23
COUP-TFII	93.55 ± 7.77	164.40 ± 19.81	↑ 1.76 ± 0.26
PPARβ/δ	44.66 ± 3.38	106.23 ± 7.94	↑ 2.38 ± 0.25
PPARγ	27.24 ± 1.74	70.25 ± 2.59	↑ 2.58 ± 0.19
Fatty acid and lipid absorption			
CD36	81.25 ± 10.72	9.99 ± 1.20	↓ 8.13 ± 1.45
FABP-3	275.84 ± 0.40	98.89 ± 0.94	↓ 2.79 ± 0.62
FABP-4	0.33 ± 0.09	0.31 ± 0.07	↓ 1.07 ± 0.38
Sugar uptake			
GLUT1	22.08 ± 6.18	35.37 ± 7.22	↑ 1.60 ± 0.55
GLUT4	0.79 ± 0.098	0.07 ± 0.004	↓ 10.83 ± 1.47
GLUT5	0.25 ± 0.030	0.19 ± 0.026	↓ 1.33 ± 0.25
Lipid catabolism			
LPL	202.40 ± 7.89	237.93 ± 27.37	↑ 1.18 ± 0.14
CPT-1	3.38 ± 0.28	3.25 ± 0.15	↓ 1.04 ± 0.10
ACS4	55.50 ± 7.33	90.78 ± 12.48	↑ 1.64 ± 0.31
AdipoR1	683.14 ± 24.07	751.89 ± 73.95	↑ 1.10 ± 0.11
AdipoR2	0.85 ± 0.09	0.21 ± 0.03	↓ 4.03 ± 0.67
PGC-1	6.79 ± 0.98	22.91 ± 0.89	↑ 3.37 ± 0.50
PDK-2	127.80 ± 11.84	69.76 ± 15.96	↓ 1.83 ± 0.45
PDK-4	25.42 ± 2.04	35.12 ± 4.87	↑ 1.38 ± 0.22
MCAD	45.50 ± 6.34	63.31 ± 8.01	↑ 1.39 ± 0.26
Energy expenditure			
UCP3	0.28 ± 0.06	0.014 ± 0.003	↓ 20.49 ± 6.09
UCP2	429.86 ± 43.04	150.23 ± 19.44	↓ 2.86 ± 0.47
Energy balance			
AMPKβ1	5.65 ± 0.74	10.79 ± 0.44	↑ 1.91 ± 0.26
AMPKγ1	120.35 ± 12.31	132.61 ± 7.26	↑ 1.10 ± 0.13
AMPKγ3	65.85 ± 7.43	10.99 ± 0.70	↓ 5.99 ± 0.78
Lipid efflux and homeostasis			
ABCA1	0.45 ± 0.19	1.21 ± 0.11	↑ 2.69 ± 1.19
ABCG1	0.21 ± 0.08	0.22 ± 0.07	↑ 1.05 ± 0.53
ApoE	108.46 ± 0.487	106.72 ± 0.628	↓ 1.02 ± 0.20
CAV1	151.63 ± 5.29	295.64 ± 32.79	↑ 1.95 ± 0.23
CAV3	698.30 ± 61.54	52.02 ± 8.09	↓ 13.42 ± 2.40
Lipid and glucose storage			
ADRP	1063.33 ± 387.40	1524.77 ± 128.29	↑ 1.43 ± 0.54
GYG1	124.30 ± 14.07	88.51 ± 9.75	↓ 1.40 ± 0.22
Lipogenesis			
SREBP-1c	0.67 ± 0.004	3.08 ± 0.041	↑ 4.57 ± 1.58
SCD-1	1684.04 ± 303.64	1626.68 ± 144.25	↓ 1.04 ± 0.21
SCD-2	521.93 ± 94.66	544.09 ± 55.77	↑ 1.04 ± 0.22
FAS	133.22 ± 24.06	177.83 ± 16.52	↑ 1.33 ± 0.27
Contractility/myogenic markers			
TNNI1	18703.82 ± 2996.83	5763.43 ± 547.03	↓ 3.25 ± 0.60
TNNI2	14573.36 ± 2143.64	4480.30 ± 507.29	↓ 3.25 ± 0.60
Myogenin	141.47 ± 14.91	290.90 ± 78.90	↑ 2.06 ± 0.60

[a] Relative expression expressed as number of transcripts per GAPDH transcript ±S.D.
[b] -Fold change ±S.D.

hypothesis suggests that a putative target tissue(s) of the sympathetic nervous system (liver, skeletal muscle, and/or white adipose) mediates the diet and β-adrenergic-induced increases in energy expenditure and thermogenesis (3). The observation that Nur77/NR4A1 is dramatically and transiently induced in skeletal muscle cells by isoprenaline, a β-adrenergic agonist, prompted us to examine the metabolic role of this receptor in skeletal muscle cells.

The expression of Nur77 in skeletal muscle, a peripheral lean tissue that has a significant role in energy expenditure, glucose disposal, and β-oxidation, led us to investigate the role of Nur77 in skeletal muscle cells with respect to the expression of genes involved in metabolism. Recent publications utilizing the C2C12 cell culture system suggest it is an ideal paradigm to investigate the regulation of lipid homeostasis in skeletal muscle (12, 13, 17, 27–30). Furthermore, our initial observation that isoprenaline treatment elicited a dramatic response in Nur77 mRNA expression coupled to the specific and selective induction of this orphan nuclear receptor (in contrast to other nuclear receptors involved in metabolism and metabolic disease) underscored the distinctive cross-talk between the adrenergic and orphan nuclear receptor pathways in skeletal muscle cells (Fig. 9). Our study demonstrates that a subgroup of genes (that includes adiponectin receptor 2, UCP3, AMPKγ3, CAV3, CD36, and GLUT4) involved in the regulation of energy balance and expenditure are dependent on the expression of Nur77. The Nur77-specific dependence of this subgroup of metabolic genes was highlighted by the observation that related genes, for example GLUT5, UCP-2, AMPKγ1, and adiponectin receptor 1, were refractory to a reduction in Nur77 expression.

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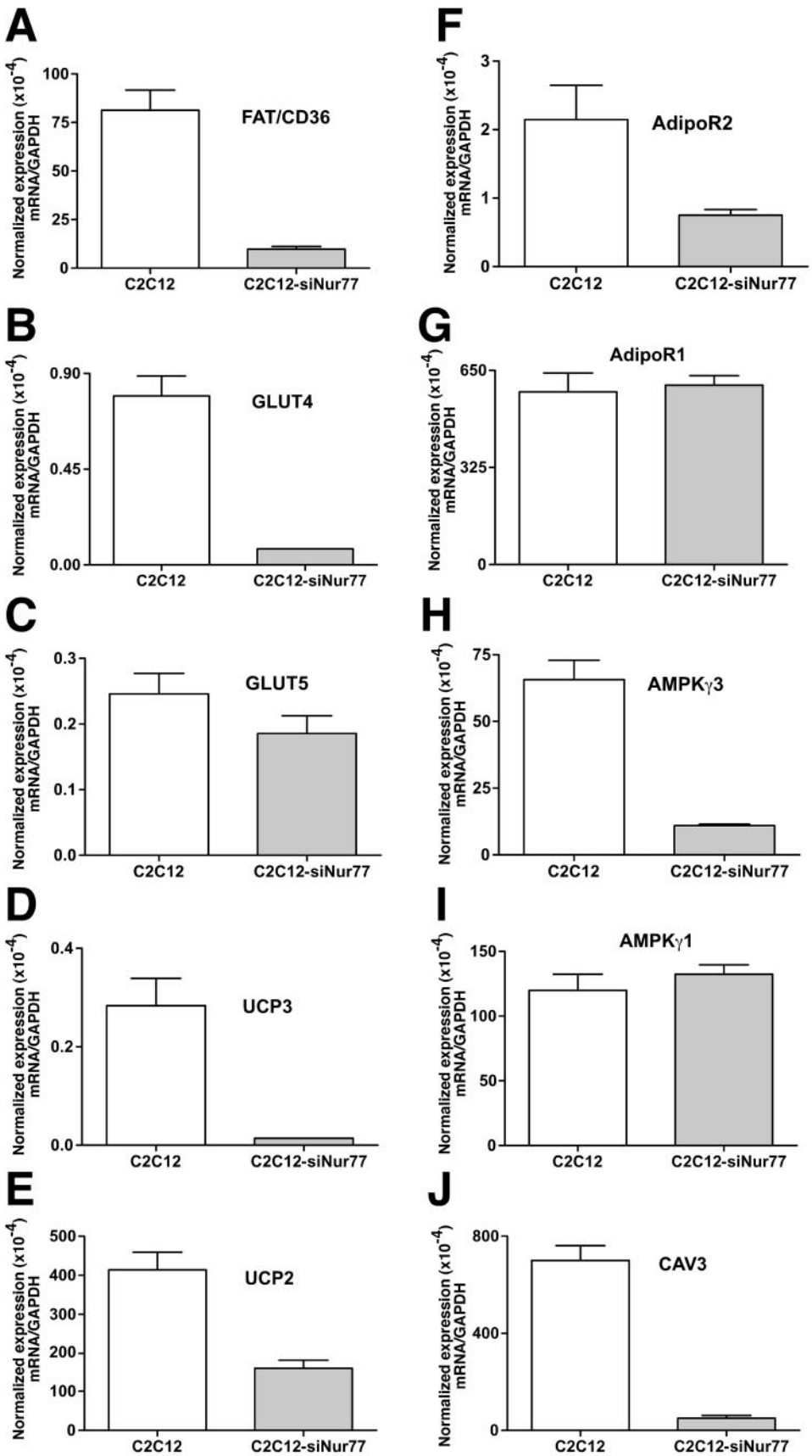

FIG. 5. **Expression profiling by Q-RT-PCR of mRNAs involved in metabolism; analyses of the wild type native C2C12 cells and the C2C12-siNur77 cells.** Q-RT-PCR analysis of endogenous mRNA expression encoding enzymes/proteins involved in lipid homeostasis, energy expenditure, etc. (described in Table I). The results were normalized and presented as the number of target transcripts per GAPDH transcript ±S.D. The category in Table I to which these genes belong is indicated. Lipid absorption, fatty acid translocase (CD36/*FAT*) (*A*); sugar uptake, GLUT4 (*B*) and GLUT5 (*C*); energy expenditure, UCP2 (*D*) and UCP3 (E); lipid catabolism, adiponectin receptor 2 (*AdipoR2*) (*F*) and AdipoR1 (*G*); energy balance, AMPKγ3 (*H*) and AMPKγ1 (*I*); lipid efflux and storage, CAV3 (*J*).

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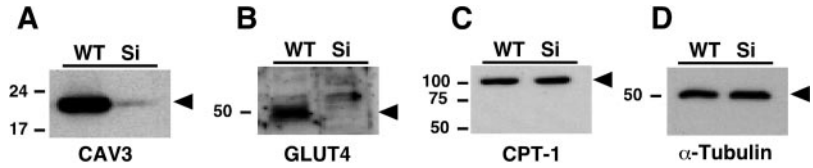

FIG. 6. **CAV3 and GLUT4 protein levels are reduced and CPT-1 protein levels remain unchanged in C2C12 and C2C12-siNur77 cells.** Total protein was extracted from C2C12 and C2C12-siNur77 cells, and 20 μg was resolved by SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes, and immunoreactive proteins were detected using a monoclonal antibody to CAV3 and polyclonal GLUT4 and CPT-1 antibodies. CAV3 (*A*), GLUT4 (*B*), and CPT-1 (C) are shown as indicated (*arrowhead*) in each panel together with the molecular mass of the resolved protein standards (in kDa). *Panel D*, α-tubulin (input control for protein loading). *WT*, wild type.

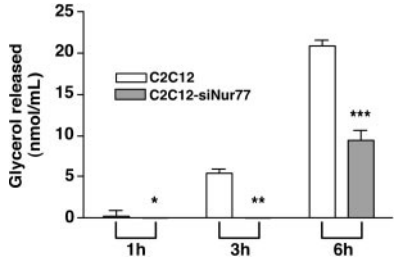

FIG. 7. **Triglyceride hydrolysis is reduced in the siNur77-expressing cells.** C2C12 cells and C2C12-siNur77 cells were differentiated for 5 days and assessed for triglyceride hydrolysis using a commercial lipolysis assay (Chemicon). Supernatants from cells incubated for 1, 3, and 6 h were assayed for free glycerol. Results are expressed as nmol of glycerol released/ml/h (mean ± S.D. of individual wells, $n = 3$). Statistical significance was assessed using Student's t test. $p < 0.05$ (*), $p < 0.01$ (**); $p < 0.001$ (***), compared with wild-type C2C12 cells.

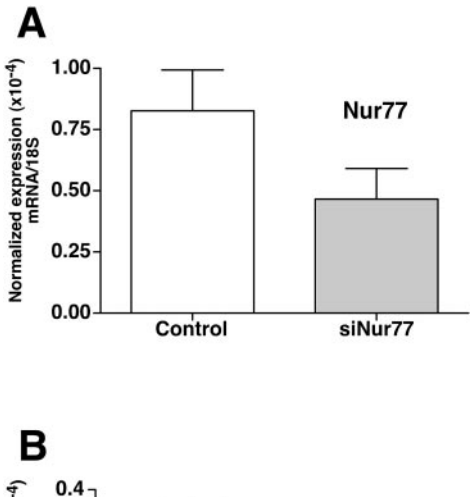

FIG. 8. **UCP3 mRNA expression is repressed in mouse muscles transiently expressing Nur77 siRNA.** Mouse tibialis cranialis muscles were injected and electroporated with a Nur77 siRNA expression vector or negative control vector. Muscles were collected 1 week later and processed for RNA, and the level of Nur77 and UCP3 mRNA expression was determined by Q-RT-PCR. Values obtained from three animals were pooled, normalized, and expressed as the number of target transcripts per 18 S transcript ±S.D.

These observations of cross-talk between Nur77 and the adrenergic pathways and the identification of this Nur77-dependent subgroup of genes is entirely consistent with the phenotype of the β-AR null/β-less mice (5), which develop severe obesity on a high fat diet due to a deficiency in diet-induced thermogenesis and energy balance.

The mice lacking all three known β_{123}-ARs (denoted "β-less mice") have a reduced metabolic rate and develop mild obesity, and these mice are unresponsive to cold and β-agonist stimulation. Oxygen consumption does not change in response to physiological cold exposure or pharmacological stimulation, and as mentioned above, the β-less mice develop severe obesity on a high fat diet. The diet-induced increase in oxygen consumption is conspicuously absent in the β-less mice, in contrast to the 20% increase in oxygen consumption observed in wild type mice after 5 days (5). Finally, linkage and association studies have implicated the β-adrenergic receptors in the accumulation of abdominal and visceral fat, which is consistent with the obesity risk in individuals with β-AR mutations, deletions, and polymorphisms (39–44).

Unfortunately, the target tissue of sympathetically mediated diet-induced adaptive thermogenesis remains obscure. Brown adipose tissue (a sympathetically innervated tissue that expresses UCP1) was thought to be the target tissue for diet-induced thermogenesis; however, the observation that the UCP1 −/− mouse phenotype is compromised for cold-induced thermogenesis but resistant to diet-induced obesity (6) has challenged this theory. Undoubtedly, β-ARs are required for diet-induced thermogenesis, and possible explanations involve UCP-2/3-dependent mechanisms and other sympathetically innervated target tissues, for example skeletal muscle, liver, or white adipose tissue (3).

In the context of this investigation a single report in the literature suggested that β-adrenergic agonists in cultured skeletal muscle cells transiently induce Nur77 mRNA expression (11). This observation was dramatically confirmed in our current study. However, the role of Nur77 in the regulation of energy balance and uncoupling in skeletal muscle (or any other major mass peripheral tissue), potential Nur77 target genes, and the mechanism of action in this metabolically demanding major mass lean tissue has remained obscure.

Our investigation has highlighted the unique and selective cross-talk between the adrenergic and orphan nuclear receptor pathways in skeletal muscle cells. Our study demonstrated that a subgroup of genes (including adiponectin receptor 2, AMPKγ3, UCP3, CAV3, CD36, and GLUT4) involved in the regulation of energy homeostasis is demonstrably dependent on the expression of Nur77 in skeletal muscle cells. Moreover, in initial animal studies we confirmed that attenuation of Nur77 expression in mouse tibialis muscle leads to reduced UCP3 expression. The relationship of this Nur77-dependent subgroup of genes is consistent with the phenotype of the obese β-AR less null mice. A number of studies have investigated ectopic and muscle-specific overexpression of UCP3 in transgenic mice and in cell culture (45–47). These investigations have reported (i) increased rates of energy expenditure, (ii) preferential lipid utilization and lipolysis, (iii) resistance to high fat diet-induced weight gain and obesity in the context of

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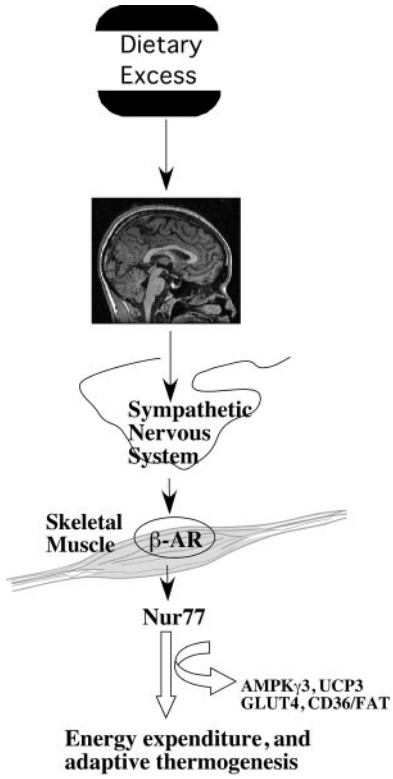

FIG. 9. **Schematic representation of the regulatory cross-talk between the adrenergic signaling pathway and the nuclear hormone receptor NR4A1/Nur77 in skeletal muscle.** The brain responds to signals from the diet, and neural infrastructure controlling energy expenditure is stimulated, which increases sympathetic activity. The nuclear hormone receptor, NR4A1/Nur77, is involved in mediating the adrenergic response in skeletal muscle. This underscores the physiological importance of regulatory cross-talk between these two critical pathways in energy homeostasis.

hyperphagic behavior, (iv) lower fasting plasma glucose and insulin levels, (v) increased glucose tolerance and clearance rate, and (vi) adaptive thermogenesis. These studies emphasize the regulatory role of UCP3 in metabolic efficiency/energy expenditure and thermogenesis and in preferential substrate utilization. We hypothesize that the effects of Nur77 agonists on skeletal muscle, a major mass peripheral tissue, would have therapeutic utility and protect against diet-induced obesity.

Similarly, we observed a reduction in the expression of the γ3 subunit of AMP-activated protein kinase in the siNur77-C2C12 cell line. This AMPK isoform is predominantly expressed in type II glycolytic muscle (48). Mutation of AMPKγ3 leads to the dramatic accumulation of glycogen in type II fast twitch white fibers, whereas AMPKγ3 null mice exhibit insulin resistance and triglyceride accumulation (33). AMPK is a critical protein kinase that operates as a metabolic stress sensor and regulator of energy balance, acting to increase fatty acid oxidation and glucose uptake and inhibit triglyceride biosynthesis (33, 49). Furthermore, recent data has suggested that the reduction in adiposity induced by adrenergic agonists involves AMPK activation in peripheral tissues (50). Our data demonstrating that the expression of AMPKγ3 mRNA is dependent on the expression of a nuclear hormone receptor that is hypersensitive to β-adrenergic stimulation is entirely consistent with the role of AMPK in the regulation of energy balance and a sensor of metabolic stress (49). Furthermore, and excitingly, our data implicate the orphan nuclear receptor Nur77 in the regulation of energy homeostasis. The coupled suppression of UCP3 and AMPKγ3 expression is also consistent with the activation of UCP3 in type II fibers by the AMPK activator, AICAR (51).

Suppressed expression of UCP3 and AMPKγ3 mRNA in the siNur77 cell line is consistent with the attenuated lipolysis observed in this cell line. This is entirely consistent with many animal studies that demonstrate muscle-specific overexpression of UCP3 and/or AMPKγ3 leads to preferential lipid utilization and increased lipolysis. Correspondingly, attenuation of UCP3 and/or AMPKγ3 expression leads to triglyceride accumulation and reduced lipolysis (33, 36, 37).

The elevated expression of SREBP-1c mRNA in the siNur77 cell line without the corresponding increases in downstream target genes involved in lipogenesis (*e.g.* fatty acid synthase, stearoyl-CoA desaturase 1 and 2) is not entirely surprising. This interesting observation, *i.e.* that the regulation of SREBP-1c may be uncoupled from fatty acid metabolism in skeletal muscle, has been reported in an LXR animal model study (17). This manuscript reported LXR-mediated activation of SREBP-1c in liver and skeletal muscle. However, the mRNAs encoding stearoyl-CoA desaturase-1 and fatty acid synthase were not induced by LXR agonist treatment in muscle (in contrast to the observations in the livers of treated mice).

We also demonstrated that GLUT4 mRNA and protein expression was significantly repressed in the C2C12-siNur77 cell line, which was in accord with the reduction in AMPKγ3 and UCP3 mRNA expression. Many studies have shown links between AMPK, UCP3, and glucose metabolism. For example, AMP kinase activators failed to induce skeletal muscle glucose uptake in the AMPKγ3 knock-out mice (33), overexpression of GLUT4 increases the expression of UCP3 in skeletal muscle (52), and muscle-specific overexpression of UCP3 leads to increased glucose tolerance and a lean phenotype despite hyperphagia (53).

The lower levels and Nur77-dependent expression of the mRNA encoding the fatty acid translocase, CD36, also correlate with the reduction of AMPKγ3 and UCP3 expression, as the β-oxidation demands in these cells would be reduced. Muscle-specific overexpression of CD36 ameliorates insulin resistance and hyperglycemia caused by dominant negative IGF-1 receptor expression. Furthermore, CD36 overexpression normalizes the fatty acid oxidation defect in these mice (54). Correspondingly, CD36 nonsense mutations lead to insulin resistance and familial type II diabetes (55), and CD36 polymorphisms have been linked to increases in free fatty acids and triglycerides (56). Interestingly, we did not observe significant changes in the expression of the genes involved in lipid catabolism and β-oxidation (*e.g.* LPL and CPT-1). However, this is not surprising as increased or decreased flux of fatty acids through the oxidation pathway does not necessarily require marked changes in genes involved in fat catabolism (45).

Finally, we demonstrated that the expression of the caveolin-3 gene and its encoded protein are attenuated dramatically in the C2C12-siNur77 cell line. Although caveolin-3 has not been directly linked to lipid regulation, studies of the non-muscle caveolin family member, caveolin-1, have suggested a role for caveolins in cholesterol transport to extracellular cholesterol acceptors such as high density lipoprotein (57, 58). Non-striated muscle caveolins 1 and 2 associate with lipid droplets in cultured cells and *in vivo* (35). Moreover, caveolin-1 null mice are resistant to diet-induced obesity (59). The studies presented here linking Nur77 expression to the regulation of caveolin-3 mRNA expression, and the Nur77-dependent nature of the other identified genes involved in the regulation of energy balance and expenditure raises the intriguing possibility that caveolin-3 may play a role during increased lipid utilization and lipolysis. This is supported by reports showing that a caveolin-3 mutant associates with lipid droplets in cultured cells (34, 35).

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The fact that the expression of this subgroup of genes regulating energy expenditure and balance is dependent on Nur77 expression, and the remarkable sensitivity of Nur77 expression to isoprenaline highlights the crucial role of Nur77 in energy homeostasis (see Fig. 9) and identifies Nur77 as a promising therapeutic target in the treatment of obesity. Skeletal muscle is rapidly emerging as a critical target tissue in the battle against obesity, type II diabetes, dyslipidemia, syndrome X, and atherosclerosis. For example, NRs such as LXR, PPARα, -β/δ, and -γ in skeletal muscle have been shown to be involved in enhancing the insulin-stimulated glucose disposal rate, decreasing triglycerides, and increasing lipid catabolism, cholesterol efflux, and plasma high density lipoprotein C levels (12, 14, 17, 31, 32). Hence, orphan NRs (for example, Nur77) that regulate energy balance in skeletal muscle have enormous pharmacological utility for the treatment of obesity. In conclusion, we suggest that in skeletal muscle cells, Nur77 programs a cascade of gene expression designed to regulate energy expenditure in this major mass lean tissue. Finally, we surmise that Nur77 agonists may have therapeutic utility in the treatment of metabolic diseases including type II diabetes and obesity.

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Nur77 Regulates Lipolysis in Skeletal Muscle Cells: EVIDENCE FOR CROSS-TALK BETWEEN THE β-ADRENERGIC AND AN ORPHAN NUCLEAR HORMONE RECEPTOR PATHWAY

Megan A. Maxwell, Mark E. Cleasby, Angus Harding, Annika Stark, Gregory J. Cooney and George E. O. Muscat

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