SIRT1 IS INVOLVED IN GLUCOCORTICOID-MEDIATED CONTROL OF UNCOUPLING PROTEIN-3 GENE TRANSCRIPTION

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Running title  SIRT1 controls uncoupling protein-3 gene

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Uncoupling protein-3 (UCP3) is a mitochondrial membrane transporter expressed preferentially in skeletal muscle. UCP3 lowers mitochondrial membrane potential and protects muscle cells against an overload of fatty acids, as well as it probably reduces excessive production of reactive oxygen species. Accordingly, UCP3 gene transcription is highly sensitive to fatty acid-dependent stimulation, but also to other unrelated stress signals. In the present study, glucocorticoids are identified as major inducers of UCP3 gene transcription in muscle. Glucocorticoids activate the transcription of the UCP3 gene through a glucocorticoid receptor-binding site in the promoter region. Glucocorticoids are capable of inducing UCP3 gene transcription independently from the myogenic regulatory factor MyoD, in contrast with the transcriptional activation of the UCP3 gene through other nuclear hormone receptors. An interplay of regulatory factors modulate positively (p300) or negatively (histone deacetylases) the action of glucocorticoids on UCP3 gene transcription via histone acetylation or deacetylation processes, respectively. Among them, SIRT1 acts as a major repressor of UCP3 gene expression in response to glucocorticoids. The action of SIRT1 requires its deacetylase activity and results in histone deacetylation in the UCP3 promoter. Moreover, it involves a specific impairment of association of p300 with the glucocorticoid receptor. Agents activating SIRT1, such as resveratrol, repress UCP3 gene expression. The control of SIRT1 activity via the cell’s metabolic redox status points to a novel regulatory pathway of UCP3 gene transcription in response to metabolic and stress signaling in muscle cells.

Uncoupling protein-3 (UCP3) is a member of the family of the mitochondrial...
membrane transporters preferentially expressed in skeletal muscle. The precise physiological function of UCP3 is still a matter of debate. Biochemical studies using isolated mitochondria indicated that the uncoupling activity of UCP3 is associated with proton permeabilization and a reduction in mitochondrial membrane potential, of which the main physiological role would be to protect against excessive production of reactive oxygen species (ROS). In fact, ROS themselves or by-products of ROS act as activators of the proton conductance activity of UCP3 (1). On the other hand, several studies have established that increased expression of UCP3 in muscle cells favors fatty acid oxidation (2;3). These observations are consistent with the transcriptional regulation of the UCP3 gene, which is extremely sensitive to fatty acids (4-6). The human and mouse UCP3 gene promoters contain peroxisome proliferator-activated receptors (PPAR)α/δ-responsive elements that mediate responsiveness to fatty acids (7;8). These same elements provide responsiveness to thyroid hormones (9). The myogenic factor MyoD acts as a permissive transcription factor for PPAR-dependent and thyroid-dependent regulation of UCP3 gene transcription in skeletal muscle (7). Although the high sensitivity of UCP3 gene transcription to fatty acids does not provide any direct evidence for the specific physiological role of UCP3, it suggests that this would be related to fatty acid handling by muscle cells. Current hypotheses are that UCP3 gene expression would be induced as a protective mechanism when levels of free fatty acids are high in order to favor fatty acid oxidation or fatty acid export from mitochondria without excess ROS production.

UCP3 gene induction may be under the control not only of stress-related signals arising from fatty acid overload or thyroid effects, but also of signals of wider origin. Thus, UCP3 mRNA is up-regulated in muscle in response to experimentally-induced sepsis (10), lipopolysaccharide (LPS) injection (11) or cancer cachexia (12) and these effects apparently occur independently of fatty acid or thyroid actions. Glucocorticoids may play a role in the induction of UCP3 gene expression in muscle in response to stress. Thus, injection of dexamethasone induces UCP3 mRNA expression (13) and glucocorticoids have been proposed to mediate most of the sepsis-mediated induction of UCP3 gene expression (10). Glucocorticoids are known to play a major role in the response of skeletal muscle to catabolic conditions such as sepsis, severe injury or burn, and glucocorticoids are considered important mediators of muscle-wasting and mitochondrial dysfunction in such conditions (14).

In the present study we identify glucocorticoids as direct activators of transcription of the UCP3 gene and we report that glucocorticoid-dependent stimulation of UCP3 gene transcription is involved in the response to LPS. Acetylation processes mediated by co-activators such as p300, which bears histone acetylase activity, as well
as deacetylation mediated by class I histone deacetylases are involved in the control of UCP3 gene transcription in response to glucocorticoids. Here we establish that the silent information regulator 2 mammalian ortholog SIRT1, is also involved in mediating negative regulation of UCP3 gene expression. SIRT1 is a member of the class III histone deacetylases that has been recently recognized as being involved in multiple biological processes (aging, caloric restriction,…) which are all associated with energy metabolism (15;16). SIRT1 is expressed in skeletal muscle and it has been implicated in myogenic differentiation (17). Thus, as SIRT1 activity is under the control of the NAD/NADH ratio, it constitutes an energy sensing molecule capable of mediating transcriptional regulation in response to the metabolic energy status of cells.

**EXPERIMENTAL PROCEDURES**

*Animals and treatments:* Male Sprague Dawley rats, 8-10 weeks of age, (Taconic) were injected intraperitoneally with LPS (Sigma, St Louis, MO) (5 mg/ kg), mifepristone (RU-486, Biomol, Plymouth, PA) (30 mg/kg), dexamethasone (Sigma, St Louis, MO) (10 mg/kg), adrenocorticotropic hormone (ACTH, Peninsula Laboratories, Belmont, CA) (1 mg/kg) or saline between 9-10 a.m. Six hours after treatment, rats were sacrificed and gastrocnemius skeletal muscle was obtained.

*Cell Culture:* Mouse myoblastic C2C12 and rat myoblastic L6 cells were obtained from the American Type Culture Collection (Manassas, VA). L6 cells were grown in DMEM (Dulbecco’s modified Eagle's medium) containing 10% fetal bovine serum (FBS) and C2C12 cells in DMEM supplemented with 10% horse serum. C2C12 cells were induced to differentiate using DMEM supplemented with 2% horse serum. Treatments with 100 nM dexamethasone, 100 μM resveratrol, 10 mM nicotinamide (NAM) (Sigma, St Louis, MO) and 50 μM sirtinol (Calbiochem, EMD Biosciences San Diego, CA) were performed in C2C12 myotubes at day 4 of differentiation.

*Analysis of UCP3 mRNA levels:* Northern blot analyses of 20 μg of total RNA from skeletal muscle samples were performed using standard methods and a fragment of the mouse UCP3 cDNA as probe (5). Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics, Image Quant software). For cells, quantitative UCP3 mRNA expression analysis was conducted using a two-step reaction and reagents from Applied Biosystems (Foster City, CA). First, 1 μg of RNA was transcribed into cDNA using TaqMan Reverse Transcription Reagents. Second, a TaqMan Real-Time PCR reaction was performed within the ABI PRISM 7700HT sequence detection system using the TaqMan Universal PCR Master Mix and the standardized primers for mouse UCP3 mRNA (Mm00494074). Appropriate controls lacking RNA, primers, or reverse transcriptase were
included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicates was used to calculate the mRNA expression. The quantity of the transcript for UCP3 in each sample was normalized to that of the endogenous reference transcript (β2-microglobulin, Mm00437762) using the comparative (2-ΔCT) method, according to the manufacturer’s instructions.

**Plasmid constructs:** The plasmid construct bearing the mouse UCP3 gene promoter from -1946 to +65 bp cloned into pGL3 basic (2mUCP3-Luc) has been described previously (9). Deleted constructs were obtained from 2mUCP3-Luc by PCR reaction using the following 5’ primers: 5’-GGGTACCGCTGGGGGAAGGGAAAGGGGGG-3’ (-615 to -594) to generate -615mUCP3-Luc; 5’-GGGGTACCTGCTTCCTAGAGATGTGG-3’ (-440 to 419) to generate -440bp mUCP3-Luc; 5’-CCTGCCTCTTGTCAAGTGATC-3’ (-100 to -79) to generate -100bp mUCP3-Luc. The complementary 3’ primer was 5’-GGGAAGCTTCCATTCCAAGGTAGCAGG-3’, +35 to +65 downstream of the transcription initiation site, according to GenBank/EMBL data (Accession AB011070). The resulting DNA products were cloned into pGL3-basic (Promega, Madison, WI), which contains the cDNA for firefly luciferase as a reporter gene. All fragments were sequenced by the dideoxy method. The point-mutation construct (2mUP3-mutGRE-Luc) and a version of the SIRT1 expression plasmid devoid of deacetylase activity through a change of histidine 363 to tyrosine were generated using a Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Complementary oligonucleotides containing the desired mutation, (5’-GCCATGATGAAAGTCTCGAGAACTAGCATGTAGTAGTTGATCAACC-3’ for 2mUCP3-mutGRE-Luc and 5’-GGATCCCTCAGTTATGATGATCAGG-3’ for SIRT1 H363Y) were used. The mutated plasmids were checked by direct DNA sequencing. The plasmid GRE-luc (18) is a vector that contains the luciferase reporter gene under the control of the consensus glucocorticoid response element (GRE).

**Transient Transfection Assays.** Transfection experiments were carried out in L6 cells at 50% confluence using FuGene Transfection Reagent (Roche Diagnostics, Barcelona, Spain). Each transfection point was assayed in duplicate or triplicate in a six-well plate and contained 1.5 µg of luciferase reporter vector, 0.3 µg of the mammalian expression vectors myc-GR (19), pCMV-MyoD (20), prSV-human TRβ1 (21), pCMV-p300 and pCMV-p300ΔHAT (22), pcM/HDAC-1 and pcM/HDAC-H141A (23), pcx-P/CAF and pcx-P/CAFΔHAT (24), pcDNA3-SRC1 (25), Tat-interactive protein-60Kda Tip60 (26), pCMV-PGC1α (27), pCMV-PGC1β (provided by A. Vidal-Puig), pCMV-SIRT1 (Upstate Biotechnology, Lake Placid, NY) or pCMV-SIRT3 (Open Biosystems, Huntsville, AL). The pRL-CMV
expression vector for the sea pansy (*Renilla reniformis*) luciferase was used as an internal transfection control (Promega, Madison, WI). Cells were incubated for 48 h after transfection and, when indicated, they were treated for 24 h before harvest with 100 nM dexamethasone or 50 nM T3. Firefly luciferase and Renilla luciferase activities were measured in a Turner Designs Luminometer (TD 20/20) using the Dual Luciferase Reporter assay system (Promega, Madison, WI). Luciferase activity elicited by UCP3 promoter constructs was normalized for variation in transfection efficiency using Renilla luciferase as an internal standard.

**Western blot analysis.** Cell extracts from myogenic cells were prepared by homogenization in a buffer containing 100 mM Tris (pH 8.5), 250 mM NaCl, 1% Igepal CA-630 (Sigma St Louis, MI), 1 mM EDTA, a cocktail of protease inhibitors (Complete-Mini, Roche Diagnostics Applied Science, Barcelona, Spain) and 0.1% phenylmethylsulphonyl fluoride. Proteins (20 µg/lane) were separated by 10% or 8% SDS-PAGE gel electrophoresis and transferred to Immobilon-P membranes (Millipore). Immunological detection was performed with specific antibodies against HDAC1 (7028 Abcam, Cambridge, UK), p300 (Santa Cruz, CA, SC-585), SIRT1 (07-131 Upstate, Lake Placid, NY), c-Myc (a gift from C. Caelles, IRB, PCB, Barcelona, Spain) or β-actin (Sigma, A5441, St Louis, MI).

**Chromatin immunoprecipitation (ChIP) assay.** L6 cells were transfected with 2mUCP3-luc or 2mUCP3GREmut-luc in the presence of the glucocorticoid receptor (GR) expression vector, which expresses a GR-Myc fusion protein, and were treated with dexamethasone (100 nM). Protein–DNA cross-linking was achieved by adding formaldehyde (final concentration 1%) for 30'. Then, 1.25 mM glycine was added. The cells were washed twice with ice-cold PBS and were centrifuged, and re-suspended in lysis buffer (50 mM Hexas-KOH pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5 mM EGTA, 0.25% Triton X-100, 0.5% Igepal) supplemented with protein inhibitors. Another centrifugation was performed and the pellets were re-suspended in wash buffer (10 mM Tris-Cl pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) and incubated for 10 min at room temperature. The samples were then centrifuged and the pellets were re-suspended in 2 ml RIPA buffer (10 mM Tris-Cl pH 8, 140 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100). The re-suspended-pellets were sonicated, centrifuged for 15' at 4°C and incubated overnight at 4°C with 5 µg of Myc antibody or 2 µg of the p300 antibody. The immunocomplex was collected by binding to 60 µl protein G-sepharose slurry. After incubation for 1 h at 4°C, the sepharose beads were collected by centrifugation and washed 5 times at 4°C with 1 ml of RIPA buffer supplemented with protein inhibitors. The pellets were then eluted in 100 µl of TE, 0.5% SDS, and 200 µg/ml proteinase K for 4 hours at 55°C. Finally, another incubation at 65°C for 6 hours was performed. After phenol
/ chloroform extraction, the DNA was used for PCR analysis.

ChIP analysis of acetyl-histone H3 and acetyl-histone H4 was performed using the supplied reagents (Acetyl-Histone H3 and H4 Immunoprecipitation, ChIP, Assay kits) from Upstate Biotechnology (Lake Placid, NY). The primers for amplifying a 690-bp fragment from -615 to + 65 bp encompassing the GRE of the mouse UCP3 promoter were 5'-GGGGTACCGCTGGGGGAAGGGAAAGGGGGG-3' (forward) and 5'-GGGAAGCTTCCATTCCATTAGGTAGCACGCAGG-3' (reverse). The PCR reaction was performed for 25 cycles at 95°C for 30 s, 66°C for 30 s, and 72°C for 1 min 30 s. The PCR products were electrophoresed in a 1 % agarose gel, visualized by ethidium bromide staining and quantified by densitometric analysis (Phoretix 1D Sofftware, Phoretix International Ltd, UK).

Immunoprecipitation Whole cell lysates were prepared by lysing L6 myoblasts in 50 mM Tris- HCl, 0.1 M NaCl, 10 mM EDTA, 1 % Nonidet P-40 (pH 7.4) containing a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). GR-Myc in whole cell lysate fraction was immunoprecipitated using a mouse monoclonal anti-Myc. 1 mg of protein was used for the immunoprecipitation. After 2 h of mixing at 4°C with 1 µg of anti-myc antibody, 40 µl of packed Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and the samples were mixed at 4°C overnight. Immunoprecipitates were washed four times with 1 ml of buffer containing 50 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, and 1 % Nonidet P-40 (pH 7.4) prior to resuspending the precipitates in sample buffer. Western-blot of immunoprecipitated extracts was performed as already indicated, except for p300 analysis in which case was used one-step IP-Western Kit (GenScript Corporation, NJ, USA), as indicated by supplier. To check for similar amounts of p300 protein in myogenic cells (p300 input), western-blot was performed using equal amounts of protein extracts before immunoprecipitation.

Adenoviral-mediated SIRT1 gene transfer. An adenoviral vector driving the expression of murine SIRT1 cDNA was constructed (Ad5-CMV-SIRT1, CEBATEG, Barcelona). C2C12 myotubes were infected with the adenoviral vector or AdCMV-GFP (control) at a multiplicity of infection of 20 or 50 for 3 h in DMEM medium. Further culture of cells was conducted with regular culture medium and conditions for the C2C12 myotubes (see above). This treatment led to an efficiency of transduction of about 90% on the basis of GFP fluorescence. Western blot analysis of SIRT1 expression was determined as described above and equal loading of protein samples was assessed by immunodetection of β-actin. C2C12 cells were studied 48 h after AdSIRT1 transduction, when the increase in the 110 kDa immunoblot signal for SIRT1 protein was around 3-4 fold with respect to controls. Statistical analyses were performed using the Student’s t test.
RESULTS

Glucocorticoids induce UCP3 gene expression in skeletal muscle. In order to determine the capacity of glucocorticoids to induce UCP3 gene expression “in vivo”, rats were treated with a single dose of dexamethasone. The results indicated a dramatic induction of UCP3 mRNA levels in skeletal muscle (Fig 1A, left). A similar result was found when rats had been injected with ACTH in order to induce a rise in endogenous glucocorticoids. Further analysis of the involvement of glucocorticoids in the control of UCP3 gene expression was performed using LPS injections. A single injection of LPS caused a dramatic induction of UCP3 mRNA levels, as previously reported (11) whereas concomitant injection with the GR inhibitor RU-486 significantly diminished the ability of LPS to induce UCP3 gene expression (Fig 1A, right). These results indicated that glucocorticoids, via the activation of GR, are involved in the control of UCP3 gene expression.

Effects of dexamethasone on UCP3 mRNA expression in myotubes. UCP3 mRNA levels were barely detectable in C2C12 myoblasts, though they became higher when C2C12 cells differentiated into myotubes. However, the UCP3 mRNA levels remained much lower than in mouse skeletal muscle (around two-orders of magnitude lower), but were nonetheless detectable using real-time PCR. Exposure of myotubes to dexamethasone led to a modest but significant induction of UCP3 mRNA levels (Fig 1B) that was in the range of that elicited by other bona-fide activators of the UCP3 gene, such as thyroid hormone T3 (9). The effect of dexamethasone was suppressed by exposure to the GR antagonist RU-486. Similar results were obtained using the rat myogenic cell line L6 (data not shown). These findings confirm the positive action of glucocorticoids on UCP3 gene expression.

Effects of dexamethasone on UCP3 promoter activity. In order to establish whether UCP3 gene induction in response to glucocorticoids is due to direct transcriptional activation, a construct containing 2kb of the UCP3 promoter region driving expression of the luciferase reporter gene was transfected into L6 myogenic cells. Addition of dexamethasone did not significantly modify basal promoter activity. However, co-transfection with an expression vector driving expression of GR conferred to the UCP3 promoter a high sensitivity to activation by dexamethasone (around 30-fold induction) (Fig 2). To date, hormonal regulation of UCP3 gene transcription by known activators such as thyroid hormones or PPAR activators required the co-transfection of a MyoD expression vector (7). Effectively, in the absence of transfected MyoD the UCP3 gene promoter remained insensitive to T3 despite the dramatic activation by glucocorticoids (Fig 2). Co-transfection with MyoD conferred to the UCP3 gene sensitivity to activation by T3 and enhanced the activity of the UCP3 promoter in the presence of transfected GR and dexamethasone. It appears therefore that glucocorticoid-
dependent regulation of the UCP3 gene, differently to other hormonal regulators, does not necessarily require MyoD. Identical results were obtained in parallel experiments using C2C12 myogenic cells (data not shown). The requirement of GR co-transfection to show responsiveness to dexamethasone was also investigated, considering the existence of endogenous GR expression in myogenic cells (28). A construct in which luciferase is driven by two copies of a consensus glucocorticoid responsive element (GRE-Luc) was transfected into L6 cells and the cells were exposed to dexamethasone in the absence or presence of co-transfected GR. The results indicated that dexamethasone significantly induced the GRE reporter construct only when GR had been co-transfected, (Fig 2). This indicated that the requirement for co-transfected GR is not a particular feature of the UCP3 gene, but was shared by standard responsiveness to glucocorticoids in this transient transfection experimental setting.

The effect of glucocorticoids on UCP3 gene transcription is mediated by a glucocorticoid-responsive element in the promoter region. In order to establish whether the action of dexamethasone is due to the presence of a GRE in the UCP3 promoter, serial deletion constructs of the mouse UCP3 gene were analyzed. The results indicated that deletion of the region from –615 to –440 in the promoter led to a drop of or around 75% in the responsiveness to dexamethasone (Fig 3A). Sequence analysis of this region revealed the presence of a site between –573 to –559 compatible with the structure of a consensus GRE (Fig 3B). A version of the UCP3 promoter construct in which point mutations were introduced in the putative GRE displayed a loss of most of the responsiveness to dexamethasone. These results indicate that most of the responsiveness of the UCP3 gene promoter to glucocorticoids relies on the presence of the -573/-559 sequence. However, the presence of minor but significant responsiveness to dexamethasone, both in the deleted construct lacking this GRE and in the mutated version, indicates that the presence of minor additional elements sensitive to glucocorticoids in the proximal region of the promoter cannot be excluded.

In order to establish whether the GRE at –573/-559 binds the GR, L6 cells were transfected with the 2mUCP3 promoter construct and co-transfected with the expression vector for a Myc-tagged version of GR. ChIP using the c-Myc antibody revealed enrichment of the 630 bp PCR product of the UCP3 promoter sequence encompassing the GRE (Fig 3C). Parallel experiments in which the transfected promoter construct corresponded to the point-mutated GRE version showed a lack of enrichment. This demonstrates that GR binds to the –573/-559 region of the UCP3 promoter.

Acetylation processes mediate the action of glucocorticoids on UCP3 gene transcription. Previous studies on the hormonal, MyoD-dependent, regulation of UCP3 gene transcription have underlined the
involvement of co-regulator-driven acetylation processes. In order to determine whether glucocorticoid-dependent activation is also mediated via acetylation processes, expression vectors for co-regulators bearing histone acetylation activity were co-transfected with the UCP3 promoter constructs and checked for their capacity to alter the responsiveness to dexamethasone. Among all the co-activator expression vectors tested (SRC-1, Tip60, PCAF, PGC-1α, PGC-1β, p300) only p300 enhanced glucocorticoid-dependent activation of the UCP3 promoter (Fig 4A) despite the fact that they were all capable of co-activating known target genes in the context of myogenic cells (see Supplementary material). Moreover, co-transfection with a mutated form of p300 devoid of histone acetyltransferase activity (p300ΔHAT), significantly reduced the extent of activation by dexamethasone, indicating that glucocorticoids were unable to fully activate transcription of the UCP3 gene in the presence of an excess of mutated p300. No effects were observed with parallel assays using a ΔHAT version of PCAF (see Supplementary material). Moreover, when cells were co-transfected with a vector driving type I histone deacetylase (HDAC1), the action of dexamethasone was severely reduced (Fig 4A). Conversely, co-transfection with a mutated form of HDAC1 in which a histidine has been mutated to aspartic acid (H141A), thus blunting deacetylase activity (23), resulted in enhancement of the action of glucocorticoids. These results indicate that acetylation processes driven by p300, and potentially down-regulated by deacetylation via type I HDACs, are involved in the mechanisms of transcriptional control of the UCP3 gene by glucocorticoids.

In order to examine this proposal directly, the UCP3 gene promoter was transfected in the presence or not of co-transfected GR plus dexamethasone, and the extent of histone-3 and histone-4 acetylation in the proximal UCP3 promoter region was determined by ChIP using anti-acetylated histone-3 or acetylated histone-4 antibodies. The results indicated that the action of glucocorticoids resulted in an increase in acetylation of histones binding to the proximal promoter region of the UCP3 gene (Fig 4B). Co-transfection with the HDAC1 expression vector reduced the extent of histone-3 and histone-4 acetylation in the UCP3 promoter and suppressed the effect driven by glucocorticoids.

**SIRT1 is involved in the acetylation-mediated processes influencing glucocorticoid-dependent activation of UCP3 gene transcription.** The experiments above indicated that, despite the repressive effects of HDAC1 on the UCP3 promoter in its response to dexamethasone, even the highest doses of co-transfected HDAC1 were unable to fully suppress the glucocorticoid effects. In order to test whether another type of histone deacetylase could be involved, cells were transfected with a vector driving expression of SIRT1, the parental type of class III histone deacetylases or sirtuins. The results
indicated that SIRT1 over-expression significantly reduced the activation of the UCP3 promoter by dexamethasone (Fig 5A). This effect was specific to SIRT1 as parallel experiments indicated that SIRT3, another member of the sirtuin family, had no effect on glucocorticoid-mediated regulation of the UCP3 gene transcription (see Supplementary material). Moreover, the effect was dependent on the deacetylase activity of SIRT1, as it was not observed when an expression vector for a mutated form of SIRT1 devoid of acetylase activity (29) (mutation of histidine 363 to tyrosine, H363Y) and driving equivalent amounts of expressed protein respect to the wild-type SIRT1 was employed for co-transfection. The enhancement of dexamethasone-induced UCP3 promoter activity by p300 was also blunted by SIRT1, whereas the repression due to p300ΔHAT was further intensified. Remarkably, co-transfection with HDAC1 plus SIRT1 expression vectors led to a full suppression of the capacity of dexamethasone to induce UCP3 promoter activity. This additive effect suggests the participation of both type of deacetylases in the control of UCP3 transcription in response to glucocorticoids.

ChIP was performed to check whether the effects of SIRT1 were associated with modifications in the acetylation status of histones binding to the UCP3 promoter region. It was observed that the induction in binding of acetylated histone-3 and acetylated histone-4 to the UCP3 promoter region when dexamethasone was present was reduced dramatically when SIRT1 was co-transfected (Fig 5B).

SIRT1 interferes with the interaction of GR and p300 in the UCP3 gene promoter. Although the action of SIRT1 in reducing the extent of histone acetylation in the UCP3 gene promoter could be attributed to its intrinsic histone deacetylase activity, it may also be the end-point of SIRT1 effects upon specific components of the GR-associated machinery that induces transcription of the UCP3 gene. The effects of SIRT1 on the interaction between GR and p300 were analyzed by immunoprecipitation in L6 myogenic cells. It was observed that SIRT1 transfection dramatically reduced such interaction, and this effect was dependent on the deacetylase activity of SIRT1, as the SIRT1H363Y mutated construct had no effect (Fig 6A). It was also determined whether SIRT1 specifically influenced the binding of p300 in association with GR, to the UCP3 gene. ChIP assays using p300 antibody indicated that GR+dexamethasone dramatically increased p300 binding to the UCP3 gene promoter. SIRT1, however, reduced such interaction (Fig 6B). The same result was observed in non-transfected cells, by amplifying the endogenous UCP3 gene (not shown). This result establishes that the repressive action of SIRT1 on glucocorticoid-mediated induction of UCP3 gene transcription involves specific interference with the recruitment of p300 to the GR interacting with the UCP3 promoter region.

The SIRT1 activator resveratrol inhibits glucocorticoid-mediated UCP3 gene
transcription. As the activity of sirtuin deacetylases is under the control of activating or repressing molecules, cells transfected with the UCP3 promoter construct were exposed to dexamethasone in the presence of resveratrol, an activator of SIRT1. The results indicated a dramatic impairment in the responsiveness to dexamethasone (Fig 7), consistent with the repressive role of SIRT1. However, potential effects of resveratrol not involving SIRT1 couldn't be excluded considering the multiple targets of action of this reagent. In any case, this was a bona-fide effect on the UCP3 promoter activity and was not due to any potential artifactual effect of resveratrol on luciferase activity (30), as other transfected plasmid constructs in which luciferase expression is driven by basal promoters (TK-Luc, for instance) were unaffected by resveratrol treatment. The effects of sirtinol and NAM, two compounds acting as SIRT1 repressors, were also determined. Although they tended to increase the responsiveness to glucocorticoids, no statistically significant differences were observed.

In order to establish whether the observed repressive effects of SIRT1 on the transfected UCP3 gene promoter occur also in the endogenous UCP3 gene, SIRT1 was over-expressed in C2C12 myotubes by the use of an adenoviral-driven SIRT1 expression vector. Enhancement of SIRT1 levels in myotubes to around 3-4-fold those of basal endogenous levels (Fig 8A) resulted in a dramatic reduction of basal UCP3 levels and completely suppressed the capacity of glucocorticoids to increase UCP3 mRNA levels (Fig 8B). Treatment of C2C12 myotubes with resveratrol also blocked glucocorticoid-dependent increases in UCP3 mRNA levels (Fig 8C). Similar results were recorded when L6 myotubes were used (data not shown).

DISCUSSION

The results of this study indicate that glucocorticoid action is a relevant component of the mechanisms of induction of UCP3 gene transcription in response to LPS and also indicate that treatment with exogenous glucocorticoid can induce “in vivo” the expression of UCP3 mRNA. This effect is dependent on GR activation, as evidenced by the inhibitory action of the GR antagonist RU-486. These observed effects of glucocorticoids in vivo are consistent with previous reports (10;13). The positive effects of glucocorticoids on UCP3 gene expression are also observed, although to a lower extent, in differentiated myogenic cells in culture.

The analysis of transcriptional regulation of the UCP3 gene indicated that glucocorticoids activate UCP3 gene transcription mostly through a glucocorticoid-responsive element (GRE) which binds GR in the UCP3 gene promoter. Remarkably, whereas all the hormonal-dependent regulation of the UCP3 gene reported to date and involving members of the nuclear hormone receptor super-family (thyroid/retnoid/PPAR) requires the action of MyoD (7;9), the effects of glucocorticoids...
via GR did not require MyoD. These findings identify a novel molecular mechanism of regulation of UCP3 gene transcription explaining several observations of UCP3 gene induction under the action of physiopathological conditions of stress upon skeletal muscle, such as the LPS treatment used in the present study or other conditions in which glucocorticoid action upon skeletal muscle is known to be enhanced (14), such as sepsis (10), burn trauma (31) or cancer cachexia (12).

Previous studies on the mechanisms of regulation of UCP3 gene transcription by fatty acids indicated that p300 plays a major role in linking PPAR-mediated fatty acid actions at the UCP3 gene promoter with acetylation of basal transcription factors and enhancement of transcription (7). The data in the present study indicate that p300 histone acetylase activity is involved in mediating the action of GR-dependent activation of UCP3 gene transcription. This is consistent with the relevance of p300 as part of the overall machinery of catabolic response in skeletal muscle to glucocorticoids (32). Moreover, the present findings indicate that a high level of UCP3 transcription in response to glucocorticoids is blunted by combined overexpression of HDAC1 and SIRT1, indicating that both type I and type III histone deacetylases are involved in the negative control of UCP3 gene transcription. Both histone deacetylases appear to be capable of diminishing the extent of acetylation of histones binding to the proximal promoter region of the UCP3 gene. Recently, HDAC1 has been reported to be capable of mediating positive effects on GR-dependent transcription of some genes (33). The present findings seem to exclude such a possibility for the UCP3 gene and indicate that, similarly to fatty-acid dependent regulation, HDAC1 is a negative regulator of UCP3 gene transcription.

The present study shows for the first time that a SIRT1 deacetylase is involved in UCP3 gene regulation. Moreover, it is shown that the repressive action of SIRT1 involves, together with potential direct effects on histone de-acetylation, effects mediated by its interaction with the GR-p300 complex. SIRT1, via its deacetylase activity, is capable of disrupting such interaction and therefore inhibiting glucocorticoid effects. This is consistent with the capacity of SIRT1 to deacetylase p300 and, by this modification, lower the capacity for trans-activation of target genes (34). However, further research will be required to establish whether GR itself can be acetylated and its transcriptional activity be influenced by such modification, similarly to that which occurs for the androgen receptor (35).

The recognition of SIRT1 as a negative regulator of the UCP3 gene in response to glucocorticoids has multiple implications in relation to the molecular mechanisms of control of UCP3 gene transcription and the physiological control of muscle cell function. First, the results of the present study constitute the first evidence that glucocorticoid-dependent gene transcription is under the negative control of the SIRT1
protein. Further research will be required to establish whether SIRT1 participates systematically in GR-mediated regulation of gene transcription. However, the preliminary data indicate only a minor effect of SIRT1 on dexamethasone-induced transcriptional activity of an artificial plasmid construct driven by a consensus GRE (unpublished observations), suggesting that SIRT1-mediated repression may be related to the particular architecture of regulatory elements in the UCP3 gene promoter region.

Second, SIRT1 has been reported to act negatively on the overall program of myogenic differentiation, including gene transcription of marker genes of late myogenic differentiation (17). The present findings are consistent in general terms with this observation, as UCP3 is a very late marker of myogenic differentiation (5,36). However, the inhibitory action of SIRT1 on UCP3 gene expression should not be viewed as a simple consequence of negative effects on myogenic differentiation, as they can take place when SIRT1 levels or activity are experimentally increased in already-differentiated myotubes. The negative action of SIRT1 on myogenic differentiation has been proposed to involve de-acetylation of MyoD, mediated indirectly via the interaction and subsequent deacetylation of the co-activator P/CAF (17). In the present case, MyoD acetylation does not appear to be the mediator of SIRT1-dependent repression. The effects of glucocorticoids on the UCP3 gene do not require MyoD and the inhibitory action of SIRT1 is observed even in L6 myogenic cells, which are devoid of MyoD (37). Recently, SIRT1 has been reported to be capable of activating the expression of several genes related to mitochondrial oxidative functions in muscle cells (38), essentially through the de-acetylation of PGC-1α, which acts as a co-activator of those genes. The fact that PGC-1α does not behave as a co-activator of the UCP3 gene may explain why there is not such positive effect, but instead an opposite, repressive effect, of SIRT1 on the UCP3 gene.

The SIRT family of histone deacetylases provides molecular mechanisms for transcriptional control in response to changes in energy metabolism, due to the control of SIRT1 activity by the NAD/NADH ratio (15,16). Mitochondrial activity and the extent of coupling is expected to influence cellular metabolism and the redox status of the cell and, for instance, UCP2 has been shown to reduce NADH levels (39). It has been recently reported that SIRT1 represses UCP2 gene transcription in pancreatic beta cells (40). UCP2 is a protein highly similar to UCP3, expressed mainly in tissues in which UCP3 is not present and, as far as currently known, having similar functions in relation to mitochondrial uncoupling and control of ROS production. The present findings showing a repressive action of SIRT1 on the expression of the UCP3 gene are consistent with a concept of a broad role of SIRT1 as a repressor of UCP genes in different tissues. Concerning the biological significance of this action, it might be hypothesized that a transient rise in the NAD/NADH ratio due to
sudden enhancement of muscle catabolism could enhance SIRT1 activity. This would therefore repress the expression of UCP3 which, in turn, would lower the NAD/NADH ratio thus providing a feed-back regulatory system prone to the maintenance of stable levels of NAD/NADH. In fact, a potential role of UCP2 and UCP3 in the control of NAD/NADH homeostasis had been suggested previously (41). Moreover, it has been proposed that SIRT1 activity is lowered when there is reduction in the NAD/NADH ratio due to a shift from carbohydrate-mediated metabolism to utilization of fatty acids (40), a metabolic situation associated with enhanced UCP3 gene transcription (4,5). Thus, the fact that negative effects of SIRT1 on UCP3 gene expression occur even in the absence of glucocorticoid-dependent stimulation raises the possibility that the negative effects of SIRT1 on UCP3 gene transcription and the modulation of this effect by the NAD/NADH ratio acts as a homeostatic mechanism in muscle cells not only in response to glucocorticoid stimulation but also as a more general mechanism. Research is under way to examine this possibility. Finally, the property of SIRT1 that it can be modulated by chemicals like resveratrol (activator) or sirtinol (inhibitor), and the concomitant effects observed on UCP3 gene expression in muscle cells, raises the possibility of exploring the use of chemicals modulating SIRT1 activity as tools to modulate UCP3 gene expression. This would promote therapeutic approaches associated with the expected biological actions of UCP3, such as reduction of intramuscular fatty acid accumulation or lowering of ROS production in muscle under acute metabolic stress conditions.

Acknowledgements: We thank Drs S. Kato, M. Martinez-Balbas, C. Caelles, M.C. Harter, M.E. Harel-Bellan, A. Vidal-Puig and P. Puigserver for kindly supplying expression vectors.

FOOTNOTES

This work was supported by Grant SAF2005-01722 (Ministerio de Educación y Ciencia, Spain).

REFERENCES


FIGURE LEGENDS

Figure 1. Effects of glucocorticoids on UCP3 mRNA expression in rat skeletal muscle and C2C12 myotubes. A, Rats were injected intraperitoneally with 5 mg/kg LPS, 30 mg/kg RU-486, 10 mg/kg dexamethasone, 1 mg/kg ACTH or equivalent volumes of saline and were studied 6 hours after treatment. B, Differentiated C2C12 myogenic cells (day 4) were treated with 100 nM dexamethasone (Dexa), 10 nM RU-486, or 50nM T3 for 24 hours. Bars are means ± SEM of 5 independent experiments and statistically significant differences with respect to controls are shown as *P<0.01.

Figure 2. Effects of dexamethasone or T3 in the presence or absence of MyoD on the UCP3 gene promoter activity. Transient transfection assays included 1.5 µg of the constructs containing 1946 bp of the mouse UCP3 (2mUCP3-Luc) promoter region or GRE-Luc as reporter genes. When indicated 0.3 µg of MyoD, thyroid hormone receptor β1 (TR) or GR expression vectors were co-transfected. Cells were treated with 100 nM dexamethasone or 50nM T3 for 24h. Results are expressed as fold induction of luciferase activity with respect to basal promoter values, and are the means ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences due to dexamethasone or T3 are shown as **P < 0.001 or *P<0.01. All samples co-transfected with MyoD-expression vector showed significantly higher differences than non-cotransfected ones (P<0.001).

Figure 3. A glucocorticoid response element between -573 and -559 bp is responsible for dexamethasone activation of the UCP3 gene promoter. A, deletion and point-mutation analysis of the mouse UCP3 promoter in response to 100 nM dexamethasone in the presence of co-transfected GR expression vector. The results are expressed as the fold-induction of luciferase activity with respect to basal promoter values, and are the means ± SEM of at least 3 independent experiments performed in triplicate. For each construct, statistically significant differences due to dexamethasone are shown as *P<0.05, and those with respect to 2mUCP3-Luc activity at a given condition as #. B, sequence of the -573 to -559 region of the UCP3 gene promoter and comparison with a consensus GRE. In lower case are shown the point-mutations introduced to generate the 2mUCP3-mutGRE-Luc construct. C, ChIP analysis of GR binding to the mouse UCP3 promoter. ChIP was performed with 2mUCP3-Luc and 2mUCP3-mutGRE-Luc constructs. The experiment was performed in the presence of co-transfected Myc-tagged GR expression vector and 100 nM dexamethasone. A representative example of ChIP analysis is shown in the left. The arrow indicates the 630 bp PCR product from UCP3 gene.
encompassing the -573/-559 region. Control + means the positive control (plasmid amplification). Bars are the means ± SEM of the fold-induction in the intensity of the amplified PCR product relative to 2mUCP3 in three independent experiments and statistically significant difference with respect to 2mUCP3 is shown as *P<0.01 or **P<0.001.

Figure 4. Effects of p300 and HDAC1 on dexamethasone-dependent activation of the UCP3 gene promoter. A, Co-transfection experiments were performed as described in Experimental procedures, and when indicated, 0.3 µg of expression vectors per well were included in the experiment. The results are expressed as fold-induction of luciferase activity with respect to basal promoter values, and are means ± SEM of at least 4 independent experiments performed in triplicate. Statistically significant differences due to dexamethasone are shown as *P<0.05 and those comparing the effect of dexamethasone between cells cotransfected or not with a given expression vector by # P<0.05. In the right, examples of Western blot analysis showing equal abundance of HDAC1 and H141, and of p300 and p300ΔHAT after transfection. B, ChIP analysis of the histone H3 and histone H4 acetylation status at the UCP3 promoter after activation with dexamethasone. Effects of HDAC1. A representative example of ChIP analysis is shown in the left. The PCR amplification product corresponds to a 630 bp fragment of the UCP3 gene promoter (see Experimental procedures). Input corresponds to the PCR of cleared chromatin before immunoprecipitation. Bars are the means ± SEM of the fold-induction in the intensity of the amplified PCR product relative to 2mUCP3 in at least three independent experiments and statistically significant difference due to GR+ dex for condition is shown as *P<0.05 and that due to HDAC-1 by # **P<0.05.

Figure 5. Effects of SIRT1 on dexamethasone-mediated activation of the UCP3 gene promoter, and functional interaction with p300 and HDAC1. A, Effect of SIRT1, SIRT1 H363Y, SIRT3, p300, p300ΔHAT and HDAC1 on the dexamethasone dependent responsiveness of 2mUCP3-Luc. Cotransfection experiments were performed as described in Experimental procedures and, when indicated, 0.3 µg SIRT1, p300, p300ΔHAT, HDAC1 or H141A expression vectors per well were included. The results are expressed as fold induction of luciferase activity with the co-transfected GR plus dexamethasone (GR + dex) compared to basal promoter values (-), and are the means ± SEM of at least 4 independent experiments performed in triplicate. Statistically significant differences due to dexamethasone are shown as
*P<0.05. and those due to the co-transfection of a given expression vector are shown as 
#P<0.05. In the bottom, examples of Western blot analysis showing equal abundance of SIRT1 
and SIRT1 H363Y after transfection are shown. B, ChIP analysis of the effects of SIRT1 on 
histone H3 and H4 acetylation status at the UCP3 promoter after activation with 
dexamethasone. A representative example of ChIP analysis is shown in the left, as in Fig 5. 
Bars are the means + SEM of the fold-induction in the intensity of the amplified PCR product 
relative to 2mUCP3 in at least three independent experiments and statistically significant 
difference due to GR+ dex for condition is shown as *P<0.05 and that due to SIRT1 by # 
**P<0.05.

Figure 6. Effects of SIRT1 on the interaction of p300 with the glucocorticoid receptor. A, 
Immunoprecipitation analysis of the interaction of GR with p300 and the effects of SIRT1. L6 
cells were transiently transfected with expression vectors for GR-myc, p300 and SIRT1 or 
SIRT1 H363Y as indicated. Cells were immunoprecipitated using an antibody directed 
against the myc-tag in the GR and Western-blotted (WB) with p300 antibody (top panel) or 
anti-myc antibody (middle panel). Whole cell extracts were probed with the p300 antibody to 
indicate equivalent p300 input before immunoprecipitation. B, ChIP analysis of the effects of 
SIRT1 on p300 binding to the UCP3 promoter after activation with dexamethasone. A 
representative example of ChIP analysis is shown in the left. The PCR amplification product 
corresponds to a 630 bp fragment of the UCP3 gene promoter (see Experimental procedures). 
Input corresponds to the PCR of cleared chromatin before immunoprecipitation. Bars are the 
means ± SEM of the fold-induction in the intensity of the amplified PCR product relative to 
2mUCP3 in the absence of GR + dex in at least three independent experiments. Statistically 
significant difference due to GR+ dex is shown as *P<0.05 and that due to SIRT1 by # 
**P<0.05.

Figure 7. Effect of resveratrol, sirtinol or nicotinamide (NAM) on the dexamethasone- 
dependent activity of the UCP3 gene promoter. Results are expressed as fold-induction of 
luciferase activity in the presence of GR and dexamethasone with respect to basal promoter 
values, and are the means ± SEM of at least 3 independent experiments performed in triplicate. 
Statistically significant differences due to 100 nM dexamethasone treatment are shown as 
*P<0.05. Statistically significant differences due to 100 μM resveratrol, 50 μM sirtinol or 10 
mM NAM treatment are shown by #.
Figure 8. Effect of SIRT1 and resveratrol on UCP3 mRNA expression in C2C12 myotubes. A, Example of an immunoblot for SIRT1 after transduction of differentiated C2C12 myotubes with 50 (+) or 100 (++) Ad-SIRT1 M.O.I. β-actin immunoblot is shown as loading control. B, Effects of dexamethasone on UCP3 mRNA levels in C2C12 myotubes over-expressing SIRT1 (50 M.O.I.). C, Effects of treatment with 100 µM resveratrol for 24h on UCP3 mRNA levels in C2C12 myotubes. Bars are means ± SEM and statistically significant differences due to dexamethasone are shown as *P<0.05, and those due to SIRT1 (B) or to resveratrol (C) as #P<0.05
Fig. 1

**A**

UCP3 mRNA
(fold increase versus saline)

- Control
- Dex
- ACTH
- LPS
- LPS+RU-486

**B**

UCP3 mRNA
(fold induction)

- T3
- Dex
- RU-486

* and # indicate significant differences compared to the respective control group.
A

GRE consensus
UCP3 GRE
UCP3 mutGRE

AGAACA NNN TGTTCT
TGATGA AAG TGTTCT
TGATGA AAG TcTcga

-573 -559

Luciferase activity (Fold induction)

0 5 10 15 20 25 30

2mUCP3-Luc (-1946bp)
-615mUCP3-Luc
-440mUCP3-Luc
-100mUCP3-Luc
2mUCP3-mutGRE-Luc

B

GRE consensus
UCP3 GRE
UCP3 mutGRE

AGAACA NNN TGTTCT
TGATGA AAG TGTTCT
TGATGA AAG TcTcga

-573 -559

C

GR-Myc Ab

- + - +

2mUCP3 2mUCP3-mutGRE Control + No DNA

Relative intensity

6 5 4 3 2 1

GR-myc-Ab - + - +

2mUCP3 2mUCP3-mutGRE

630 bp
Fig 5

A

SIRT1 SIRT1 H363Y SIRT3 SIRT1 + p300 SIRT1 + p300ΔHAT SIRT1 + HDAC1

2mUCP3-Luc activity (fold induction)

SIRT1 SIRT1 H363Y

SIRT1 Ab

110kDa

B

2 mUCP3- Luc

Anti-acetyl-Histone H3

Input

GR + dex

Anti-acetyl-Histone H4

Input

Relative intensity

GR + dex

A

Myc-GR+ p300

\[ \begin{array}{ccc}
- & - & SIRT1 \\
- & SIRT1 H363Y \\
WB: Anti p300 & & \\
WB: Anti Myc-GR & & \\
p300 input & & \\
\end{array} \]

B

2 mUCP3- Luc

\[ \begin{array}{c}
GR + dex: \\
SIRT1: \\
\end{array} \begin{array}{ccc}
- & + & + \\
- & - & + \\
\end{array} \]

Anti-p300

Input

Relative intensity

\[ \begin{array}{cc}
GR + dex: & - & + & + \\
SIRT1 & - & - & + \\
\end{array} \]

Anti-p300

\[ \begin{array}{c}
* \\
# \\
\end{array} \]
Fig 7

2mUCP3-Luc activity (fold induction)

- Resveratrol
- Sirtinol
- NAM

GR + dex

* #
**Fig 8**

A. Western blot analysis showing the expression of SIRT1 and β-actin. Following treatment with Ad-GFP or Ad-SIRT1, a significant increase in SIRT1 protein levels is observed (110 kDa). Similar levels of β-actin (41 kDa) are detectable in both treatments.

B. Bar graph demonstrating the effect of Dexamethasone on UCP3 mRNA levels in cells transfected with GFP or SIRT1. Treatment with Dexamethasone results in a significant increase in UCP3 mRNA levels in SIRT1-treated cells compared to GFP controls.

C. Bar graph showing the effect of Resveratrol on UCP3 mRNA levels in cells treated with Dexamethasone. Resveratrol treatment leads to a significant induction of UCP3 mRNA levels in cells treated with Dexamethasone.