Evidence for Nr4a1 as a cold-induced effector of brown fat thermogenesis

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Kanzleiter, Timo, Tatjana Schneider, Isabel Walter, Florian Bolze, Christoph Eickhorst, Gerhard Heldmaier, Susanne Klaus, and Martin Klingenspor. Evidence for Nr4a1 as a cold-induced effector of brown fat thermogenesis. Physiol Genomics 24: 37–44, 2005. First published October 11, 2005; doi:10.1152/physiolgenomics.00204.2005.—Acute cold exposure leads to norepinephrine release in brown adipose tissue (BAT) and activates uncoupling protein (UCP1)-mediated nonshivering thermogenesis. Chronic sympathetic stimulation is known to initiate mitochondrial biogenesis, UCP1 expression, hyperplasia of BAT, and recruitment of brown adipocytes in white adipose tissue (WAT) depots. Despite distinct functions of BAT and WAT in energy balance, only a few genes are exclusively expressed in either tissue. We identified NUR77 (Nr4a1), an orphan receptor, to be induced transiently in brown adipocytes in response to β-adrenergic stimulation and in BAT of cold-exposed mice. Subsequent reporter gene assays demonstrated an inhibitory action of NUR77 on basal and peroxisome proliferator-activated receptor (PPAR)γ-retinoid X receptor (RXR)α-mediated transactivation of the Ucp1 enhancer in heterogeneous cotransfection experiments. Despite this function of NUR77 in the control of Ucp1 gene expression, nonshivering thermogenesis was not affected in Nur77 knockout mice. However, we observed a superinduction of Nor1 in BAT of cold-exposed knockout mice. We conclude that NUR77 is a cold-induced negative regulator of Ucp1, but phenotypic consequences in knockout mice are compensated by functional redundancy of Nor1.

NUR77 is a potent effector of NUR77 in other cell types, we compared

nerve growth factor-induced gene B; Nor1; Ucp1

IN SMALL MAMMALS, brown adipose tissue (BAT) is the primary organ involved in thermoregulatory heat production. The underlying biochemical mechanism known as nonshivering thermogenesis (NST) depends on the uncoupling protein UCP1, which short circuits mitochondrial respiration and ATP synthesis in brown adipocytes. In cold-exposed or overfed animals, this mechanism enables regulated dissipation of proline-tive force as heat (47, 53). The dense sympathetic innervation regulates thermogenic activity and respiratory capacity of BAT (for review, see Ref. 10). The release of norepinephrine from sympathetic nerve endings in response to cold exposure leads to an immediate stimulation of lipolysis and increased oxygen consumption. Successively, the sympathetic nervous system trigger a well-characterized recruitment process that leads to BAT growth and mitochondrial biogenesis (for reviews, see Refs. 29 and 38). Recruitment is mainly mediated by β-adrenergic receptors and involves increased proliferation of precursor cells (4, 5) and differentiation of brown adipocytes (22, 51) but also a decrease in the number of cells undergoing apoptosis (36). Many studies have demonstrated that recruitment involves altered expression of numerous genes in BAT with considerable knowledge accumulated on transcriptional control of the Ucp1 gene (for review, see Ref. 11). Transcriptional activation of the Ucp1 gene occurs within hours in response to cold exposure or β-adrenergic stimulation (7). The Ucp1 enhancer directly responds to cAMP response element-binding protein (CREB) but also harbors functional response elements for several nuclear receptors, namely thyroid receptor, retinoic acid X receptor (RXR), and peroxisome proliferator-activated receptor (PPAR), which are coactivated by PPARγ coactivator-1 (PGC1) (7).

We aim to identify genes in brown adipocytes immediately responsive to β-adrenergic stimulation and analyze the function of these genes with respect to nonshivering thermogenesis. In the search for genes differentially expressed in brown adipocytes in response to β-adrenergic stimulation, we found a strong induction of NUR77. Nur77 is an immediate early gene and constitutes, together with Nor1 and Nur1, the Nr4a family of orphan nuclear receptors (40). Nur77 is induced in different cell types by serum growth factors, phorbol esters, membrane depolarization, fatty acids, cAMP-dependant signaling, and androgens (17, 24, 35, 43, 52, 54, 59), but expression in brown adipocytes has not been reported. Pertaining to the role of retinoic acid signaling in the control of UCP1 transcription (49), it is interesting that Nur77 has been shown to modulate retinoid signaling by heterodimerizing with the RXR in JEG-3 and PC12 cells (26, 48).

Diverse biological functions have been assigned to the members of the Nr4a family. Nur77 has been demonstrated to play a crucial role in T-cell receptor-mediated apoptosis (37, 61, 62). It also regulates expression of the propiomelanocortin- and corticotropin-releasing factor genes in the anterior pituitary and the steroid-21-hydroxylase gene in the adrenal gland (60), suggesting a function in the neuroendocrine control of the hypothalamo-pituitary-adrenal axis (44). Furthermore, Nur77 mRNA expression is upregulated by a variety of stimuli controlling cell proliferation, differentiation, and apoptosis in different cell lines and tissues (40).

We investigated the time course of Nur77 gene expression in HIB-1B brown adipocytes in response to β-adrenergic stimulation and in BAT of warmth- and cold-exposed mice, and further explored tissue specificity of the cold response. In cultured brown adipocytes, we analyzed the effect of β-adrenergic stimulation on the immunoreactive Nur77 protein mass. We then directly tested the possible role of Nur77 in the regulation of Ucp1 gene expression using reporter gene assays and chromatin immunoprecipitation (ChIP). Because nerve growth factor (NGF), a secretory product of brown adipocytes, is a potent effector of Nur77 in other cell types, we compared

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the effect of β-adrenergic stimulation and NGF treatment on subcellular localization of NUR77 in HIB-1B brown adipocytes. Finally, we studied Nur77+/− mice with respect to thermogenic capacity of BAT and possible compensation of loss of function by Nnor1 and Nurrl.

MATERIALS AND METHODS

Cell culture. HIB-1B cells were grown on cell culture dishes and provided with medium (DMEM/F12, Gibco BRL) and 10% FCS (Biochrom), with a change of medium on every third day. After confluence, FCS was reduced to 7% and insulin (17 nM, Sigma) was added to promote differentiation. On day 9 after confluence, cells were differentiated to mature brown adipocytes and either treated with isoproterenol (100 nM, Sigma) or NGF (100 ng/ml, rat recombinant β-NGF; Sigma), with the duration of treatment indicated in the figure legends. To test whether hormonal effects (isoproterenol and NGF) on subcellular localization depend on active translation, cells were treated in parallel with cycloheximide (180 μM, Merck) in some experiments.

cDNA representational difference analysis. cDNA representational difference analysis (cDNA-RDA) was performed according to published protocols (23) with minor modifications (30). To identify β-adrenergically regulated transcripts, cDNAs were synthesized from isoproterenol-treated as well as control HIB-1B cells and subjected to cDNA-RDA. In parallel experiments, cDNA from isoproterenol-stimulated cells was employed as a tester and as a driver to isolate transcripts either up- or downregulated by the stimulus. The cDNA fragments represented after two rounds of subtractive hybridization were cloned using the pGEM-T vector (Promega) and analyzed for differential expression of their corresponding mRNA by Northern blot analysis.

Northern blot analysis. Cells were harvested at 11 time points after addition of isoproterenol ranging from 5 min to 24 h. Medium was removed and dishes immediately frozen at −70°C. Total RNA was isolated using the guanidium thiocyanate method (9). Tissue samples were kept at −70°C before RNA preparation following the Trizol RNA isolation protocol (Invitrogen), modified by introducing a second precipitation step as described previously (57). Isolated RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically at 260/280 nm (Genequant, Amersham Biosciences). Equal amounts of total RNA (20 μg) were run on 1% denaturing agarose gels, checked for RNA integrity and loading by ethidium bromide staining, and blotted on nylon membranes (Hybond-N, Amersham Biosciences). All probes were random prime labeled with [32P]dCTP (Rediprime, Amersham Biosciences). Nur77 mRNA was detected with the 417-bp Nur77 fragment obtained by cDNA-RDA. Norl mRNA was detected with a 265-bp PCR fragment corresponding to 2,106–2,499 bp of the cDNA-RDA. Norl cDNA (GenBank accession no. D38530). Detection of Nurrl mRNA was performed with a 393-bp PCR fragment corresponding to 2,106–2,499 bp of the rat Norl cDNA (GenBank accession no. NM_013613). Ucp1 mRNA was detected using a full-length rat cDNA (3). Hybridization was performed overnight in 0.5 M sodium phosphate buffer containing 1% BSA, 7% SDS, and 1 mM EDTA at 64°C after prehybridization of the blots in the same buffer for at least 1 h.

Immunocytochemistry. HIB-1B cells were cultured on glass slides (Menzel, Germany) as described above. After treatment with either isoproterenol or NGF in the presence or absence of cycloheximide, cells were immediately fixed and pretreated for immunodetection as described (28). Nur77 protein was detected using a monoclonal mouse Nur77 antibody (Ref. 13; kindly supplied by Tim Fahrner) in combination with a second cysteine-5 (Cy5)-conjugated goat anti-mouse-IgG antibody (Jackson Laboratories). Slides were incubated with antibodies for 1 h at room temperature; diluted in PBS buffer, pH 7.5, containing 0.5% BSA, 1% gelatin, 0.1% Tween 20, 1% Triton X-100, and 2% goat serum; and then washed in washing buffer (PBS, pH 7.5, 1% Triton X-100). Nuclear counterstaining was performed employing BOPRO-3 (5 μM diluted in PBS, pH 7.5; Molecular Probes). After incubation for 1 h, slides were washed in washing buffer and dipped in distilled water. Cells were fixed in Entellan (Merck) and sealed with coverslides. Immunofluorescence was detected with a confocal laser scanning microscope (Leica Tcs SP2). Images were analyzed using ImageJ 1.23 (Wayne Rasband, National Institutes of Health) supplemented with a Leica SP-reader plug-in. The ratios of the Nur77 signal intensities detected in the cytosol and in the nucleus were calculated from 10 fluorescence intensity measurements per cell with 10 cells at each time point and treatment. This ratio was then expressed in relation to untreated control cells.

Western blot analysis. HIB-1B nuclear extracts were prepared from β-adrenergically stimulated and control cells (41), and protein concentrations were determined using the Bradford assay. After sample precipitation in acetic acid, 50 μg of nuclear protein extracts were loaded on a 10% SDS-polyacrylamide gel (32) and transferred to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences) in a semi-dry electrophoretic chamber. Successful protein transfer was controlled by Ponceau staining of the membrane. Nur77 protein detection was carried out using the monoclonal mouse Nur77 antibody (13). The membrane was first incubated in blocking solution (PBS, pH 7.5, 0.05% Tween 20, 1% casein, 20% FCS) for 1 h at room temperature and then incubated overnight with the Nur77 antibody at 1:50 dilution in PBS, pH 7.5, and 0.05% Tween 20 at 4°C. The membrane was then probed with a goat anti-mouse-IgG-horseradish peroxidase-conjugated secondary antibody (Biomol) at a 1:2,000 dilution for 1 h at room temperature and developed by enhanced chemiluminescence (Supersignal, Pierce).

Western blot analysis of Nur77-transfected HEK293 cells was performed as above except for the primary antibody: in this experiment, we used a monoclonal Nur77 antibody from PharMingen.

Gene expression in BAT of cold-exposed mice. We then investigated the time course of Nur77 gene expression in brown and white adipose tissue of cold-exposed mice. All animal experiments in this publication had been approved by the local government authorities (VI63-19c/20/15cMR17/1). Male NMRI mice (Harlan-Winkelman) were housed in a 12:12-h light-dark cycle at room temperature (23°C), with food and water ad libitum. Before cold exposure, mice aged 7–9 wk were single caged for at least 1 wk and individually transferred to precooled cages supplied with a minimum of wood shavings at 5°C. The duration of cold exposure ranged from 0 min to 7 days (0 min, 10 min, 1 h, 4 h, 8 h, 1 day, 7 days), with three mice per treatment group. At the end of exposure, mice were exposed to CO2 and killed by heart incision between 8:00 AM and 12:00 PM (Central European Time). Dissected tissues were snap-frozen in liquid nitrogen for subsequent RNA extraction. RNA was extracted as described above. Furthermore, tissue distribution of Nur77 expression was investigated in mice either kept at room temperature or exposed to 5°C for one h (N = 5 each).

Luciferase assays. The UCP1 enhancer was amplified by PCR (forward, 5′-CAT CTT AAG AGA AGA GCT CGG ACA C-3′; reverse, 5′-CAT CTT AAG AGA AGA GCT CGG ACA C-3′) using a template corresponding to GenBank accession no. 63418 (kindly donated by L. Kozak). Restriction sites for SacI/BgIII flanking the UCP1 enhancer were introduced by modified primers, and the resulting PCR fragment was cloned into the pGL3 vector (Promega). The pGL3 plasmid bears simian virus 40 promoter (SV40) upstream of the cDNA-encoding photinus luciferase. The promotorless reporter gene vector pRhG-B (Promega) expressing the Renilla luciferase was cotransfected in all experiments as an internal control for transfection efficiency. HEK293 cells were transfected using ProFection Kit (Promega), according to the manufacturer’s instructions. Cells in a 12-well dish with 30–60% confluence were treated with 15 ng of experimental vector, 200 ng of control vector, and 500 ng of each expression vector. The total amount (2 μg) of introduced DNA was kept constant by adding empty expression vector (pcDNA3). The cells were har-
vested 48 h after transfection. Luciferase measurement was performed using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The results were normalized by Renilla luciferase activity.

Chromatin immunoprecipitation assays with the Ucp1 enhancer. HIB-1B adipocytes were stimulated with 100 nM isoproterenol for 1 h and chemically cross-linked thereafter with 1% formaldehyde. ChIP assays were conducted with the Chromatin Immunoprecipitation Assay Kit from Upstate according to the manufacturer’s instructions, using a monoclonal Nur77 antibody (PharMingen), a polyclonal RXRα antibody (Santa Cruz), and a polyclonal cytochrome-c antibody (New England Biolabs), respectively.

The same primers used for the construction of the reporter construct were employed for real-time PCR amplification of the Ucp1 enhancer from the chromatin immunoprecipitates. Fragments were amplified on an iCycler (BioRad) using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer’s instructions (annealing at 58°C). Samples were quantified in triplicates and resulting mean values expressed in relation to cytochrome-c values.

Basal oxygen consumption and nonshivering thermogenesis in Nur77−/− mice. We investigated whether ablation of Nur77 in mice has an effect on nonshivering thermogenesis. Mice carrying the Nur77 knockout allele on a C57BL/6 background (33) were supplied by Jeff Milbrandt (Washington University, St. Louis, MO). They were provided with standard rodent chow (Altromin 1314, Lage) and water ad libitum and bred at room temperature (23°C) in a 12:12-h light-dark cycle at our institute. Mice were genotyped at the age of 4 wk by Southern blot analysis as described previously (33) and caged individually 2 wk before the onset of experiments. At the age of 8–10 wk, mice were acclimated to the climate chamber was reduced to 23°C to protect mice from maximum. NST capacity was calculated by subtracting basal oxygen consumption over 12 min and calculated as the rate of oxygen consumption maintained over 4-min intervals for 2–3 h. Basal oxygen consumption was determined at thermoneutral ambient temperature (30°C) with oxygen consumption recorded in 4-min intervals for 2–3 h. Basal oxygen consumption was defined as the lowest level of oxygen consumption maintained over 12 min and calculated as the mean of four successive data points. Nonshivering thermogenesis was stimulated by a single subcutaneous injection of 1.0 mg/kg norepinephrine, which elicits the maximal thermogenic capacity of BAT in mice (15). Before the injection of norepinephrine, ambient temperature in the climate chamber was reduced to 23°C to protect mice from heat stress. Oxygen consumption was recorded in 20-s intervals. Sustained maximal heat production for >140 s was taken as the NST maximum. NST capacity was calculated by subtracting basal oxygen consumption.

Finally, cold-acclimated mice and mice from an age-matched control group kept at room temperature (3 males and 3 females of each genotype) were killed to determine wet weight of BAT and also liver, lung, spleen, heart, testis, and skeletal muscle. No differences between male and female mice were observed.

In a separate experiment, the cold-induced regulation of Nor1 mRNA expression in BAT was compared in Nur77−/− and wild-type mice. Total RNA was isolated from interscapular BAT and analyzed by Northern blotting.

Statistical analysis. Data were expressed as means ± SE when appropriate. Reporter gene assay data and Nor1 gene expression in BAT of Nur77 KO mice were analyzed by one-way ANOVA followed by post hoc pairwise comparisons (Holm-Sidak test). The effect of ambient temperature and genotype on metabolic parameters in Nur77 KO mice was tested by repeated measures two-way ANOVA. In all tests, significance level was set to $P < 0.05$.

RESULTS

Nur77 gene expression in HIB-1B cells. Analysis of differential gene expression in HIB-1B cells in response to stimulation with the β-adrenergic agonist isoproterenol revealed ~200 cDNA fragments. Among the first cDNA clones sequenced we found a 417-bp fragment corresponding to the 3′-UTR of Nur77 mRNA (identical to nt 1,890–2,306 in GenBank accession no. J041113). Differential expression of Nur77 was confirmed by Northern blot analysis of the original RNA samples used for cDNA-RDA as well as new samples from control and isoproterenol-treated HIB-1B brown adipocytes.

On the basis of this initial observation, we first investigated the time course of β-adrenergic stimulation. This revealed a very strong stimulation of Nur77 mRNA in response to isoproterenol (Fig. 1A). In control cells, Nur77 mRNA was expressed at a low level and was clearly detectable with extended film exposure time (not shown). In response to isoproterenol, Nur77 mRNA level rapidly increased to a maximum within 1 h and then returned to control level. Nur77 mRNA was already elevated 20 min after addition of the agonist and could be detected up to 4 h after stimulation (Fig. 1A). Compared with Nur77, the response of Ucp1 mRNA level to β-adrenergic stimulation was slower (Fig. 1A). Maximal expression of Ucp1 mRNA was attained 4 h after the addition of isoproterenol (Fig. 1A).

Immunodetection of Nur77. Nuclear extracts of HIB-1B cells were prepared to further investigate the effect of β-adrenergic stimulation (isoproterenol) on Nur77 protein. In extracts prepared from untreated cells, the monoclonal Nur77 antibody detected a distinct band at ~62 kDa (Fig. 1B). Isoproterenol treatment transiently increased immunoreactive protein mass, with maximal signal intensity observed 1.5 h after stimulation. However, a large fraction of the immunoreactive protein mass shifted to a higher molecular size ranging...
from 66 to 80 kDa. This gel mobility shift of NUR77 immunoreactive mass was reversed 2 h after stimulation and diminished successively with time.

The same shift in immunoreactive mass could be observed in protein extracts of Nur77-transfected HEK293 cells. In mock transfected cells, no signal could be obtained (Fig. 1B).

**Cold-induced increase of Nur77 gene expression in BAT.** We then examined the physiological regulation of Nur77 mRNA in BAT and white adipose tissue (WAT) of cold-exposed mice, a condition known to stimulate norepinephrine release from sympathetic innervation of BAT and WAT. In warmth-acclimated mice, Nur77 mRNA was expressed at a low level in BAT but was hardly detectable in WAT. In cold-exposed mice, a transient upregulation of Nur77 expression was observed in BAT but not in WAT (Fig. 2A). The maximal expression level of Nur77 mRNA in BAT of cold-exposed mice was attained within 1 h, matching the response observed in cultured HIB-1B cells stimulated with isoproterenol (Fig. 1A). After seven days of cold exposure, Nur77 mRNA levels tended to be lower than in control animals.

**Tissue specificity.** Differential regulation of Nur77 expression in BAT and WAT prompted the analysis of gene expression in other tissues to clarify whether the cold-induced rise in Nur77 mRNA was restricted to BAT. Nur77 mRNA levels were compared in a panel of tissues from warm- and cold-exposed mice (Fig. 2B). We confirmed differential regulation of Nur77 mRNA in BAT and WAT of cold-exposed mice. Among the other tissues, Nur77 mRNA was found at high abundance in brain, lung, testis, and skeletal muscle. Weaker expression was observed in heart, spleen, and hypothalamus. Cold-induced regulation of Nur77 mRNA was not found in any of these tissues excluding the heart. Nur77 mRNA level in the heart was strongly increased in response to 1 h of cold exposure. No signals were detected in liver and kidney.

**Reporter gene assays with the Ucp1 enhancer.** Regulatory influence on the Ucp1 enhancer by NUR77 was tested in reporter gene assays. The reporter construct was activated by cotransfection with PPARγ and RXRα (2- to 3-fold), thereby confirming functionality of the assay. NUR77 significantly decreased activation by PPARγ/RXRα. Even transfection of NUR77 alone decreased the basal reporter activity (Fig. 3A). This experiment was repeated four times.

**Interaction of Nur77 with the Ucp1 enhancer.** We performed ChIP assays to investigate interaction of NUR77 with the Ucp1 enhancer in brown adipocytes. To ensure the functionality of the assay, the known interaction of RXRα with the Ucp1 enhancer (50) was tested in parallel and as a negative control a cytochrome-c antibody was applied. Interaction of RXRα...
with the Ucp1 enhancer could be shown in three independent experiments (Fig. 3B), but NUR77 did not interact with the Ucp1 enhancer. Treatment of the cells with isoproterenol (1 h) before ChIP assays did enhance interaction of RXR/H9251 with the Ucp1 enhancer.

Subcellular localization of NUR77. We compared the effects of isoproterenol and NGF stimulation on subcellular localization of NUR77 protein. In untreated HIB-1B cells, NUR77 immunofluorescence was exclusively located in the nucleus, which was verified by co-staining with BOPRO-3 (Fig. 4). In response to both isoproterenol and NGF, a rapid but transient rise in NUR77 immunofluorescence intensity was observed in the cytoplasm in relation to the nucleus. To illustrate this observation, we compared the ratios of immunofluorescence intensities in the cytoplasm and the nucleus in relation to untreated cells. The transient increase of this ratio in response to isoproterenol was completely abolished when translation was inhibited by cycloheximide (Fig. 4). In contrast, the increased cytosolic distribution of NUR77 observed in response to NGF was not altered by cycloheximide (Fig. 4).

Phenotypic analysis of Nur77 knockout mice. Body mass and basal oxygen consumption were similar in wild-type mice and in mice heterozygous or homozygous for the Nur77 knockout allele (Table 1). Ablation of Nur77 did not impair the acute stimulation of maximal nonshivering thermogenesis elicited by norepinephrine. Cold acclimation for 2 wk increased basal oxygen consumption (+20%) and NST capacity (+65%) in all mice, irrespective of genotype. Mean BAT and heart wet weight were increased in cold-acclimated mice, but comparison by genotype revealed no difference (Table 1). All other dissected tissues were not affected by treatment or genotype (data not shown).

The absence of phenotypic consequences of Nur77 ablation with respect to nonshivering thermogenesis and a report on functional redundancy of Nur77 and Nor1 (8) prompted us to investigate the expression of all Nr4a family members in BAT.

Table 1. Comparison of body mass, basal O2 consumption and NSTcap, and BAT and heart wet weight

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<th>23°C</th>
<th>2 wk at 5°C</th>
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<td></td>
<td>+/+</td>
<td>+/-</td>
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<tr>
<td>Body mass, g</td>
<td>27.2±2.0</td>
<td>27.5±1.9</td>
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<tr>
<td>Basal O2 consumption, ml/h</td>
<td>32.8±3.0</td>
<td>31.7±0.8</td>
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<tr>
<td>NSTcap, ml/h</td>
<td>122.1±4.7</td>
<td>124.4±9.3</td>
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<tr>
<td>BAT, mg</td>
<td>74.5±8.3*</td>
<td>75.5±8.5*</td>
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<tr>
<td>Heart, mg</td>
<td>126.3±6.4*</td>
<td>136.7±10.7*</td>
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Comparison of body mass, basal oxygen consumption and nonshivering thermogenesis capacity (NSTcap), and brown adipose tissue (BAT) and heart wet wt in wildtype (+/+), mice and mice heterozygous (+/-) or homozygous (-/-) for the Nur77 knockout allele. Body mass, basal oxygen consumption, and NSTcap were increased significantly by cold acclimation but not affected by genotype (P < 0.01, 2-way ANOVA for repeated measurements). BAT and heart mass were elevated by cold (P < 0.001, 2-way ANOVA). *Tissues were dissected from an age-matched control group kept at 23°C for comparison with cold-acclimated mice.
Nor1 mRNA level in response to 1-h cold exposure (4°C) was increased in adipocytes as well as in BAT of mice exposed to cold, but not in WAT.

Notably, cold-induced Nur77 expression also occurs in the heart. This could be explained by increased cardiac output in cold-exposed mice. It is known that mechanical stimulation of blood vessels induces expression of Nr4a family members (39).

Adrenergic induction of Nur77 gene expression has been previously demonstrated in the heart (56), in cultured rat astrocytes, and in rat cerebral cortex (1, 2) as well as the mouse C2C12 skeletal muscle cell line (34, 35, 42), but to our best knowledge has not been reported in brown adipocytes.

On the protein level, immunocytochemistry and Western blot results demonstrate that increased Nur77 mRNA levels in response to β-adrenergic stimulation are reflected by increased Nur77 synthesis. In unstimulated cells, Nur77 resides in the nucleus (Fig. 4) and appears as a distinct band on Western blots, corresponding to the molecular mass of 62 kDa as expected from the coding sequence (Fig. 1B). Isoproterenol treatment causes an increase of immunoreactive Nur77 mass in nuclear extracts with a simultaneous shift in molecular size up to 80 kDa. Previously, it was reported that, in PC12 cells, treatment with NGF (13, 16) results in phosphorylation of Nur77 (nerve growth factor-induced gene B; NGFI-B) at multiple sites, leading to nuclear export (25), loss of DNA binding, and decreased transcriptional activity (21, 25). In brown adipocytes, Nur77 may also be modified by phosphorylation, but this alone can hardly explain the observed mass mobility shift. Interestingly, two small ubiquitin-like modifier (SUMO) consensus sites found in NURR1 (14) are conserved in Nur77 as well as in NOR1; however, direct SUMOylation remains to be demonstrated.

As the increase of Nur77 gene expression in HIB-1B adipocytes stimulated with the β-adrenergic agonist preceded the rise in Ucp1 mRNA levels, we investigated a possible function of Nur77 in Ucp1 gene transcription. Indeed, the upstream enhancer element essential for BAT-specific expression and noradrenergic regulation of the Ucp1 gene in vivo (31) contains an almost perfect NBRE. We therefore tested whether Nur77 as a transcription factor activates this enhancer in luciferase reporter gene assays. In the heterologous HEK293 cell system, where Nur77 is not present, we observed a two- to threefold increase of luciferase activity in cells cotransfected with PPARγ/RXRα, corresponding to previous reports in HIB-1B cells (55). Surprisingly, Nur77 consistently blunted this PPARγ/RXRα-mediated transactivation of the Ucp1 enhancer. Thus, despite rapid cold-induced expression in BAT, this nuclear receptor rather inhibits Ucp1 gene expression.

Interestingly, Maxwell et al. (42) reported a positive regulation of UCP3 by Nur77 in skeletal muscle and discuss a possible role of Nur77 and UCP3 for diet-induced thermogenesis. However, after cold exposure, we could not observe an increase of Nur77 expression in skeletal muscle, and the putative role of UCP3 for thermoregulatory heat production in skeletal muscle has been challenged (46).

The inhibitory action of Nur77 must be indirect, as our ChIP analysis excludes binding of Nur77 to the enhancer (Fig. 3B). It has been demonstrated that Nur77/RXRα heterodimers are translocated from the nucleus to the cytosol after treatment of PC12 cells with NGF (26). Notably, NGF is secreted by brown adipocytes in response to adrenergic stimulation (45). We therefore suggest that competition for heterodimer formation and nuclear export of Nur77/RXRα in brown adipocytes cause the inhibitory effect, as retinoid signaling is known to be crucial for Ucp1 gene expression (26, 49).

To test this possibility, we compared subcellular Nur77 distribution in brown adipocytes in response to isoproterenol and NGF. Cytoplasmic localization of Nur77 was increased in response to isoproterenol as well as NGF (Fig. 4). For isoproterenol, the increase was most likely due to de novo synthesis of Nur77, as the rise in cytoplasmic staining was...
abolished in translationally inactive cells. In contrast, NGF treatment increased cytoplasmic localization, even when translation was blocked. Notably, in HIB-1B adipocytes treated with NGF, we did not observe induction of Nur77 mRNA and Nur77 protein levels (not shown). Therefore, increased cytoplasmic localization in response to NGF must be due to nuclear export of Nur77. Until now, NGF had been regarded as a neurotrophic factor for sympathetic neurons innervating BAT, but our results demonstrate a novel autocrine activity of NGF on brown adipocytes. In resemblance to NGF-mediated nuclear export of Nur77 as a heterodimer with RXRα in PC12 cells, in brown adipocytes, transient removal of RXRα from the nucleus could limit the extent of Ucp1 gene expression in response to sympathetic activation.

To further explore the role of Nur77 for thermogenic function of BAT, we compared the capacity of nonshivering thermogenesis in wild-type mice and mice bearing a Nur77 knockout allele. No evidence for altered capacity of nonshivering thermogenesis was found in Nur77−/− mice, neither at room temperature nor in the cold-acclimated state. Basal oxygen consumption was also unaffected by genotype. Nur77−/− mice have been studied previously with respect to multiple phenotypes, including the control of T-cell apoptosis, but no phenotypic disorders were found (27, 33). However, in transgenic mice, targeted overexpression of a dominant-negative form of Nur77, which blocks activity of all Nr4a family members in T cells (8), caused deficient clonal deletion of self-reactive thymocytes (6, 62). Therefore, the ablation of Nur77 in knockout mice may be compensated by the other members of the Nr4a family, Nor1 and Nur1 (12). In particular, Nor1 and Nur1 are able to activate the same response elements and heterodimerize with the same factors as Nur77. Accordingly, the observed cold-induced upregulation of Nor1 and Nur1 gene expression in BAT of Nur77−/− mice may compensate for the lack of Nur77. Nor1 expression was even augmented in knockout mice after cold exposure, which further supports compensatory function suppressing phenotypic consequences.

In summary, transient cold-induced expression of Nur77, inhibition of the Ucp1 enhancer, and augmented expression of Nr4a family members in BAT of cold-exposed Nur77 knockout mice suggest that this orphan nuclear receptor may be a negative regulator of thermogenic capacity in BAT. Recruitment of capacity for nonshivering thermogenesis is rather costly and therefore only desirable in mice experiencing prolonged cold exposure. We speculate that transient expression of Nur77 in BAT of mice may function to suppress Ucp1 expression during short-term cold exposure. This would perfectly fit with previous reports that brown fat growth in mice depends on the duration of daily cold exposure, and the recruitment of nonshivering thermogenic capacity only occurs when cold exposure exceeds 2 h/day (18, 19, 58). Given the suggested inhibitory action on Ucp1 gene expression, the observed downregulation of Nur77 in mice chronically exposed to cold (7 days) would facilitate maintenance of a high thermogenic capacity.

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