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Norepinephrine and rosiglitazone synergistically induce *Elovl3* expression in brown adipocytes

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Jörgensen JA, Zadravec D, Jacobsson A. Norepinephrine and rosiglitazone synergistically induce *elovl3* expression in brown adipocytes. *Am J Physiol Endocrinol Metab* 293: E1159–E1168, 2007. First published September 14, 2007; doi:10.1152/ajpendo.00213.2007.—The *Elovl3* gene, which putatively encodes for a protein involved in the elongation of saturated and monounsaturated fatty acids in the C20–C24 range, is expressed in murine liver, skin, and brown adipose tissue (BAT). In BAT, *Elovl3* is dramatically upregulated during tissue activation in response to cold exposure, and functional data imply that ELOVL3 is a critical enzyme for lipid accumulation in brown adipocytes during the early phase of tissue recruitment. The activation of BAT is controlled by sympathetic nerve activity and norepinephrine release. By using primary cultures of brown adipocytes, we show here that the induced *Elovl3* gene expression is synergistically regulated by norepinephrine and the peroxisome proliferator-activated receptor (PPAR) γ ligand rosiglitazone. In addition, the potency of rosiglitazone to induce *Elovl3* expression was several orders of magnitude higher than for the PPAR α and PPAR δ ligands WY-14643 and L-165041, respectively. The maximal increase in mRNA level by norepinephrine and rosiglitazone is achieved by induced transcription as well as increased mRNA stability, and the whole process requires novel protein synthesis. We conclude that norepinephrine and PPAR γ , despite having different roles in brown adipocyte activation and differentiation, cooperate in expanding the intracellular lipid pool by synergistically stimulating *Elovl3* expression.

fatty acid synthesis; fatty acid elongation; very long-chain fatty acids; lipid metabolism; peroxisome proliferator-activated receptor- γ

DE NOVO FATTY ACID SYNTHESIS is performed by fatty acid synthase (FAS), which uses acetyl-CoA and malonyl-CoA to generate fatty acid up to 16 carbons in length (56). Subsequently, these fatty acids can undergo additional modifications by enzymes localized in the endoplasmic reticulum, i.e., desaturation and/or elongation (20, 25).

Elovl3 is a member of the *Elovl* gene family, which code for enzymes involved in the first step (condensation reaction) of fatty acid elongation from (C16) into long-chain or very long-chain fatty acids (VLCFA). The *Elovl* family consists of at least six members in mouse and human that are believed to perform substrate-specific fatty acid elongation with respect to chain length and degree of unsaturation (15, 23, 24, 30, 53, 59). The *Elovl3* gene, of which the encoded protein has been suggested to regulate the elongation of saturated and monounsaturated fatty acid in the C20–C24 range, is significantly expressed in liver, skin, and brown adipose (BAT; see Refs. 19, 53, 58).

Elovl3 was initially identified as a gene involved in BAT recruitment upon its highly elevated mRNA expression in BAT following cold exposure (52). The recruitment of BAT is controlled by the release of norepinephrine from sympathetic nerve endings (9, 51) and results in increased thermogenic capacity of the organ (10, 33). Along with BAT recruitment, the synthesis of several mRNA species involved in energy expenditure and fatty acid metabolism, including the uncoupling protein 1 (UCP1) and *Elovl3*, are induced. Recent data from our laboratory obtained from studies on *Elovl3*-ablated mice suggest that the ELOVL3 protein has a function in BAT to synthesize saturated and/or monounsaturated VLCFA for triglyceride formation to generate lipid droplets predominately during the early phase of tissue recruitment (57).

In differentiated cultures of brown adipocytes, a mixture of norepinephrine, the synthetic glucocorticoid dexamethasone, and the peroxisome proliferator-activated receptor (PPAR)- α ligand Wy-14643, which rendered the adipocytes in a high oxidative state, was shown to be required for substantial induction of *Elovl3* expression (19). A PPAR α regulation of *Elovl3* expression in brown adipocytes was confirmed both in primary cultured cells obtained from PPAR α -ablated mice and in cold-exposed animals (19).

PPARs are ligand-activated transcription factors regulating genes involved in lipid metabolism (1, 11, 16, 21, 22, 43, 60). The three different PPAR subtypes (PPAR α , PPAR β/δ , and PPAR γ) regulate different processes in the cell. For example, PPAR γ has been shown to be a master regulator of adipocyte differentiation in vitro (36, 41, 42), whereas PPAR α activation results in increased expression of genes involved in fatty acid oxidation.

Whereas the role of PPAR γ and PPAR α in adipose tissue is well studied, the role of PPAR δ is less understood. In skeletal muscle, PPAR δ has been shown to regulate fatty acid oxidation and development (27, 47). However, in adipose tissue, PPAR δ is believed to control preadipocyte proliferation (14) and to promote adipocyte differentiation by inducing PPAR γ expression (4, 5, 29).

The PPAR subtypes show different tissue distribution; however, brown adipose tissue (BAT) is a unique example of a tissue that coexpresses high levels of all three PPARs (7, 55).

We here examined the role of different PPARs on *Elovl3* expression in primary cultured brown adipocytes to gain more insight into how ELOVL3 controls fatty acid synthesis and lipid accumulation in BAT. We found that the PPAR γ ligand rosiglitazone, in conjunction with norepinephrine and dexamethasone treatment, was the most potent of all the

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ligands tested. Furthermore, our data show that the norepinephrine- and rosiglitazone-induced *Elovl3* expression is controlled both on a transcriptional level and on the level of mRNA stability.

MATERIAL AND METHODS

Chemicals. L-Norepinephrine (NE) bitartrate (Arterenol), dexamethasone, WY-14643, cycloheximide, and actinomycin D were obtained from Sigma. Rosiglitazone was purchased from Alexis and L-165041 from Calbiochem. NE, cycloheximide, and actinomycin D was dissolved in water, L-165041 was dissolved in dimethyl sulfoxide (DMSO) while all other compounds were dissolved in ethanol according to the manufacturer's instructions.

Animals. Mice (10 wk old with a body wt of ~30 g) of the NMRI strain (B&K, Stockholm, Sweden) were divided into three groups (three mice in each) and injected intraperitoneally at room temperature (~21°C) with norepinephrine (3 μ mol/kg body wt), rosiglitazone (30 mg/kg body wt), or vehicle (25% ethanol), respectively in a total volume of 100 μ l.

Primary cell culture of brown adipocytes. Brown adipocyte precursor cells were isolated from interscapular, axillary, and cervical BAT depots of 3-wk-old male NMRI mice (B&K) and grown in culture, as described earlier (31). Briefly, tissues were minced in HEPES-buffered solution containing 0.18% (wt/vol) collagenase type

II (Sigma), and cells were digested for 30 min at 37°C. The digest was filtered through a 250- μ m nylon filter to remove undigested parts, followed by a 15-min incubation on ice to allow mature cells and lipid droplets to float. Thereafter, the infranatant was filtered through a 25- μ m nylon membrane. Precursor cells were collected by centrifugation (700 g for 10 min), washed in DMEM (Invitrogen), pelleted (700 g for 10 min), and resuspended in 0.4 ml culture medium/mouse.

Cells were seeded in six-well plates (Corning) and grown in culture medium consisting of DMEM (1g glucose/l) supplemented with 10% newborn calf serum, 4 nM insulin, 25 μ g/ml sodium ascorbate, 10 mM HEPES, 4 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin at 37°C in an atmosphere of percent CO₂ in air. On day 1, cells were washed in DMEM, and fresh medium was added; this was followed by medium change on day 3 and 6. Administration of chemicals was performed as described in the legends for Figs. 1–9, and the cells were harvested on day 9, if not stated otherwise.

RNA analysis. Cells were harvested in 1 ml of Ultraspec solution (NordicBiosite), and total RNA was isolated according to the procedure described by the manufacturer. Total RNA (10 μ g) was separated by electrophoresis on an ethidium bromide-containing agarose-formaldehyde gel and blotted to a HybondXL membrane (GE Healthsciences). Prehybridization was carried out for 2–4 h, and hybridization was performed overnight in 50% formamide, 5 $\times$ Denhart's solution, 50 mM sodium phosphate, pH 6.5, 0.5% SDS, and 100 μ g/ml herring sperm DNA at 42°C. A cDNA fragment for *Elovl3* (53)

Fig. 1. Peroxisome proliferator-activated receptor (PPAR)-regulated *Elovl3* mRNA expression in cultured brown adipocytes. Northern blot showing *Elovl3* mRNA levels. Primary cultures of brown adipocytes were grown for 9 days. From day 6 to 9, cells were treated with 0.1 μ M norepinephrine (N), 1 μ M dexamethasone (D), and 0.01 μ M–30 μ M of the PPAR α ligand WY-14643 (A), the PPAR δ ligand L-165041 (B), or the PPAR γ ligand rosiglitazone (C). Total RNA was extracted and analyzed as described in the experimental section. RNA (10 μ g) was used for each lane, and the blots were hybridized with an *Elovl3* cDNA probe. D: RNA levels were quantified and normalized to 18S. The mean value from norepinephrine-dexamethasone treated cells was set to 100%, and all other values were expressed relative to this. E: *Elovl3* mRNA levels in brown adipose tissue (BAT) in mice injected with vehicle (C), norepinephrine (NE), or rosiglitazone (R) and analyzed after 18 h by Northern blot and quantitative PCR ($n = 3$ experiments).

was [32 P]dCTP labeled using Ready-to-go labeling beads (GE Healthcare), according to the manufacturer's instructions, and used as a probe. Membranes were washed two times in $2\times$ saline-sodium citrate (SSC) and 0.1% SDS at room temperature for 15 min and one time in $0.1\times$ SSC and 0.1% SDS at 42°C for 20 min. The hybridized membranes were put on phosphorimaging plates for 48–72 h and analyzed in a FLA-300 reader (Fuji). 18S was detected using a 473-nm laser in the FLA-3000 reader and used for normalization. Blots were quantified using Fuji Film Science Lab software and statistically analyzed by repeated-measures ANOVA and Tukey's multiple-comparison test in GraphPad PRISM. For quantitative PCR, 500 ng total RNA was reverse transcribed by reverse transcription reagents (Applied Biosystems, Foster City, CA) with random hexamer primer in a total volume of 20 μl . RT-qualitative PCR aliquots with 4 μl of the sample cDNA were mixed with TaqMan Universal Mastermix (Applied Biosystems), SYBR GREEN PCR Master Mix, 18S probe (18S; HS99999901_S1), and *Elovl3* primer with diethyl pyrocarbonate H_2O to a final volume of 26 μl /well and were measured in triplicate for each sample. Expression was analyzed in an ABI Prism 7000 sequence detection system. Data were normalized to 18S. The primer sequence was as follows: *Elovl3* forward 5'-GCCTCTCATCCTCTGGTCT-3' and *Elovl3* reverse 5'-TGCCATAAACTTCCACATCCT-3'.

RESULTS

Rosiglitazone is a potent inducer of Elovl3 expression in brown adipocytes. We have shown earlier that induction of *Elovl3* expression in brown adipocytes requires a combination of both norepinephrine and glucocorticoid exposure for 3 days after the cells have reached confluence (52). Previous studies also show that enhanced lipolysis by PPAR α activation positively regulates *Elovl3* expression in BAT (19). To examine whether any of the other PPAR isoforms were also able to influence *Elovl3* expression, primary cultures of brown adipocytes were grown until confluence (day 6) and treated for 3 days with norepinephrine and dexamethasone plus the PPAR α ligand WY-14643, the PPAR δ ligand L-165041, or the PPAR γ ligand rosiglitazone at different concentrations. These compounds have all been shown to be ligands of their respective PPAR isoform, although with different potency (13, 45).

As seen in Fig. 1, A, B, and D, both the PPAR α and the PPAR δ ligand required relatively high concentrations, $>1\text{ }\mu\text{M}$, to induce *Elovl3* expression. However, rosiglitazone induced a sixfold increase in *Elovl3* expression at $0.1\text{ }\mu\text{M}$ (Fig. 1, C and D). In addition, we also found that rosiglitazone, in similarity with norepinephrine, is a potent inducer of *Elovl3* expression in BAT when injected in mice (Fig. 1E). Based on this, we examined the nature of the PPAR γ and the adrenergic regulation of *Elovl3* expression in brown adipocytes in more detail.

Enhanced Elovl3 expression coincides with increased brown adipocyte differentiation. Primary brown adipocytes spontaneously differentiate at $\sim$ day 5–6 in culture. Norepinephrine stimulation induces expression of genes associated with increased thermogenic capacity such as UCP1 (18, 37, 38); adrenergic stimulation thus brings the differentiation process to completion. However, the intensity of the norepinephrine effect varies depending on the differentiation status of the cell; in the preadipocyte (day 4 and earlier), norepinephrine is unable to induce UCP1 gene expression, whereas the greatest response can be seen in differentiated cells around day 6. In adipocytes kept in culture for 10 days or more, the ability of the cells to respond to norepinephrine, e.g., stimulated UCP1 gene expres-

sion, is reduced (38). PPAR γ ligands have been shown to promote adipocyte differentiation, including UCP1 expression in brown adipocytes (44, 46, 49, 50). Because the PPAR γ ligand rosiglitazone is a potent stimulator of *Elovl3* expression, we wanted to examine the effect of rosiglitazone on *Elovl3* expression along with differentiation of the brown adipocyte.

Primary cultures of brown adipocytes were grown for 4, 5, or 6 days and thereafter treated with norepinephrine, dexamethasone, and rosiglitazone for 3 days. As can be seen in Fig. 2, *Elovl3* mRNA levels increased with time in culture, showing the highest levels on day 9. Rosiglitazone augmented the effect of norepinephrine and dexamethasone, resulting in a 10-fold increase compared with the norepinephrine- and dexamethasone-treated cells. Based on these results, further studies were performed on day 9.

Synergistic induction of Elovl3 expression by norepinephrine, rosiglitazone, and dexamethasone. Earlier studies showed a synergistic action of norepinephrine and dexamethasone on *Elovl3* expression in primary cultures of brown adipocytes (52). To examine the effect of rosiglitazone in combination with norepinephrine and dexamethasone, cells were treated with norepinephrine-dexamethasone, rosiglitazone alone, norepinephrine-rosiglitazone, dexamethasone-rosiglitazone or all three compounds together.

Exposing cells to norepinephrine or dexamethasone alone did not give detectable *Elovl3* expression levels, as described earlier (19; data not shown). However, rosiglitazone alone induced the expression to a similar level as norepinephrine and dexamethasone combined (Fig. 3). In addition, together with rosiglitazone, both norepinephrine and dexamethasone had a synergistic effect on *Elovl3* expression. The greatest level of expression was achieved with the combined norepinephrine-dexamethasone-rosiglitazone treatment.

Norepinephrine and rosiglitazone induce Elovl3 expression to a maximum within 1 day of treatment. In BAT, where the physiological control of BAT activity is executed through norepinephrine, *Elovl3* expression has been shown to peak between 24 and 72 h of cold exposure (52). Because both norepinephrine and rosiglitazone significantly induced *Elovl3*

Fig. 2. Enhanced *Elovl3* mRNA expression in brown adipocytes during differentiation. Brown adipocytes were treated with norepinephrine-dexamethasone (ND) and norepinephrine-dexamethasone-rosiglitazone (NDR) on the days indicated (3 days in total) and analyzed for *Elovl3* mRNA as described in Fig. 1. Results are means $\pm$ SE from 3 independent experiments performed in duplicate, normalized to 18S RNA; the value from day 6–9 cells treated with NDR was set to 100%.

Fig. 3. Synergistic effect of norepinephrine, rosiglitazone, and dexamethasone on *Elovl3* mRNA expression. Brown adipocytes were cultured for 9 days. Cells were treated for 3 days with 0.1 μ M norepinephrine and 1 μ M dexamethasone (ND), 1 μ M rosiglitazone (R), norepinephrine and rosiglitazone (NR), dexamethasone and rosiglitazone (DR), or all three compounds together (NDR) and analyzed for *Elovl3* mRNA expression as described in Fig. 1. Results are means $\pm$ SE from 2–4 independent experiments performed in duplicate, normalized to 18S RNA. Data from NR-treated cells are from a separate experiment ($n = 2$), and data from DR-treated cells are also included in Fig. 4 ($n = 4$). The value from NDR-treated cells was set to 100% in all experiments, and the other values were expressed relative to this.

expression in cultured cells, we wanted to examine the time frame within which *Elovl3* expression can be induced by these two activators. Cells were treated for three days with either norepinephrine/dexamethasone, rosiglitazone/dexamethasone, or only dexamethasone, complemented with norepinephrine alone, rosiglitazone alone, or together, respectively, 6 h, 1 day, or 3 days before harvest.

As seen in Fig. 4A, *Elovl3* mRNA was already significantly elevated after 6 h of rosiglitazone treatment; however, maximal expression was measured after a 24-h treatment. This is within the same time frame as has been measured for other PPAR γ target genes, such as aP2 and UCP1 (35, 42, 48).

The time of induction of *Elovl3* mRNA expression by norepinephrine was similar to rosiglitazone-induced expression (Fig. 4B), except that the relative induction was lower than for rosiglitazone-treated cells mainly because of the high basal expression after rosiglitazone-dexamethasone pretreatment. A 6-h treatment resulted in significantly elevated mRNA levels, whereas maximal induction was observed after 24 h.

Addition of norepinephrine and rosiglitazone together also resulted in an increase of *Elovl3* expression within 6 h of

treatment, reaching a maximal level after 1 day (Fig. 4C), similar to what was seen when the two agents were added separately. This suggests that norepinephrine and rosiglitazone together with dexamethasone cooperatively stimulate the cultured brown adipocytes to reach a maximal *Elovl3* mRNA level within 24 h, similar to what is seen in vivo during cold exposure.

Fig. 4. Time course of norepinephrine and rosiglitazone effect on *Elovl3* expression. Primary cultures of brown adipocytes were treated as described below and analyzed for *Elovl3* mRNA expression. A: cells were treated for 3 days with norepinephrine and dexamethasone. Rosiglitazone was added 3 days, 1 day, or 6 h before harvest. B: cells were pretreated for 3 days with dexamethasone and rosiglitazone 3 days, 1 day, or 6 h before harvest; norepinephrine was added to the cells. C: cells were treated with dexamethasone for 3 days. Norepinephrine and rosiglitazone were added simultaneously, at the same time points as for A and B. Results are means $\pm$ SE from 4 independent experiments (except for dexamethasone in C where $n = 2$) performed in duplicate (normalized to 18S RNA). Treatment with NDR for 3 days was set to 100% and is the same for all three experiments.

Norepinephrine- and rosiglitazone-induced Elovl3 expression is a transcriptional event that requires novel protein synthesis. The induction of *Elovl3* expression could occur either through an active transcription of the *Elovl3* gene or through increased mRNA stability or both. To examine this, cells stimulated with either rosiglitazone-dexamethasone or norepinephrine-dexamethasone for 3 days were treated with the transcription inhibitor actinomycin D together with either norepinephrine or rosiglitazone, respectively, for 9 h before harvest and then analyzed.

Norepinephrine treatment resulted in significantly increased *Elovl3* mRNA levels after 9 h (Fig. 5A), which is in agreement with previous results. However, this increase was totally abolished in the presence of actinomycin. Rosiglitazone treatment also resulted in significantly increased *Elovl3* mRNA levels, which, similarly to norepinephrine-induced *Elovl3* expression, was inhibited in the presence of actinomycin (Fig. 5B). Actinomycin alone did not significantly affect *Elovl3* expression. These results suggest that both the norepinephrine-induced and the rosiglitazone-induced expression of *Elovl3* is a transcriptional event.

To examine if the stimulation of *Elovl3* expression is also dependent on induced protein synthesis, rosiglitazone-dexamethasone or norepinephrine-dexamethasone stimulated cells were exposed to the protein synthesis inhibitor cycloheximide together with either norepinephrine or rosiglitazone, respectively, 9 h before harvest.

As seen in Fig. 6, A and B, both norepinephrine and rosiglitazone treatment resulted in a significant increase in *Elovl3* expression, which was inhibited by cycloheximide. The addition of cycloheximide alone resulted in a reduction of *Elovl3* mRNA levels. This was most prominent in rosiglitazone- and dexamethasone-pretreated cells where the mRNA level was reduced to ~60% of control cells.

These results show that protein synthesis is required for both norepinephrine- and rosiglitazone-induced *Elovl3* expression.

Norepinephrine, rosiglitazone, and dexamethasone treatment induces stabilization of Elovl3 mRNA. The above data show that norepinephrine and rosiglitazone, in the presence of dexamethasone, stimulate *Elovl3* expression on a transcriptional level, at least as a first event. Our data also show that, although norepinephrine and rosiglitazone were able to stimu-

Fig. 5. The transcriptional inhibitor actinomycin blocks norepinephrine- and rosiglitazone-induced *Elovl3* mRNA expression. Primary cultures of brown adipocytes were treated as described below and analyzed for *Elovl3* mRNA expression. A: cells were treated for 3 days with dexamethasone and rosiglitazone. Before harvest (9 h), norepinephrine, alone or in combination with actinomycin, was added. B: alternatively, the cells were treated for 3 days with norepinephrine and dexamethasone. Before harvest (9 h), rosiglitazone, alone or in combination with 1 μ g/ml of the transcriptional inhibitor actinomycin, was added. As a control, actinomycin was also added alone. Results are means $\pm$ SE from 4 independent experiments performed in duplicate (normalized to 18S RNA). The value at time 0 h was set to 100%.

Fig. 6. The protein synthesis inhibitor cycloheximide blocks the norepinephrine and rosiglitazone induction of *Elovl3* expression. The experiment was performed as in Fig. 5 but with the protein synthesis inhibitor cycloheximide. Primary cultures of brown adipocytes were treated as described below and analyzed for *Elovl3* mRNA expression. **A**: cells were treated for 3 days with dexamethasone and rosiglitazone. Before harvest (9 h), norepinephrine, alone or in combination with cycloheximide, was added. **B**: alternatively, the cells were treated for 3 days with norepinephrine and dexamethasone. Before harvest (9 h), rosiglitazone, alone or in combination with 50 μ M of cycloheximide, was added. As a control, cycloheximide was also added alone. Results are means $\pm$ SE from 4 independent experiments performed in duplicate (normalized to 18S RNA). The value at time 0 h was set to 100%.

late *Elovl3* expression up to maximal mRNA levels within 24 h, the cells require exposure to a combination of norepinephrine, rosiglitazone, and dexamethasone for $\sim$ 3 days to reach maximal level.

To further elucidate mechanisms behind the norepinephrine-dexamethasone-rosiglitazone treatment, we examined the half-life of the *Elovl3* transcript either in the presence or in the absence of norepinephrine-dexamethasone-rosiglitazone combined with the transcription inhibitor actinomycin.

On day 6 of culturing, cells were treated for 3 days with the norepinephrine-dexamethasone-rosiglitazone mixture to induce *Elovl3* expression. Before harvest (9 h), the media was exchanged and norepinephrine-dexamethasone-rosiglitazone was either readded or withdrawn.

Removal of the norepinephrine-dexamethasone-rosiglitazone treatment resulted in decreased mRNA levels of $\sim$ 25%, whereas the readddition of the mixture led instead to a slight increase in *Elovl3* expression (Fig. 7).

Surprisingly, the addition of actinomycin for 9 h did not significantly affect *Elovl3* mRNA levels in either the presence or absence of norepinephrine-dexamethasone-rosiglitazone stimulation (Fig. 7), indicating that the norepinephrine-, dexamethasone-, and rosiglitazone-stimulated increase in *Elovl3*

mRNA level is not only dependent on transcription. Because withdrawal of the norepinephrine-dexamethasone-rosiglitazone mixture, but not inhibition of transcription, reduced the *Elovl3* mRNA level, it rather suggests that *Elovl3* mRNA degradation is prevented in the presence of norepinephrine-dexamethasone-rosiglitazone. However, from our data, it is not clear whether all three components are necessary for this.

The *Elovl3* mRNA half-life in the absence of norepinephrine-dexamethasone-rosiglitazone was estimated to be 20–25 h.

Increased Elovl3 mRNA stability is dependent on novel protein synthesis. To further investigate whether the norepinephrine-, dexamethasone-, and rosiglitazone-induced mRNA stability is dependent on protein synthesis, cells were stimulated as before for 3 days with norepinephrine-dexamethasone-rosiglitazone and cycloheximide, or actinomycin was added 9 h before harvest. The presence of cycloheximide decreased the *Elovl3* mRNA by 50% compared with norepinephrine-, dexamethasone-, and rosiglitazone-treated cells (Fig. 8). A similar comparison in the presence of actinomycin did not result in decreased *Elovl3* expression, indicating that the increased mRNA stability by norepinephrine-dexamethasone-rosiglitazone requires novel protein synthesis.

Fig. 7. NDR treatment influences *Elovl3* mRNA stability. Primary cultures of brown adipocytes were cultured for 9 days. The last 3 days in culture, cells were treated with norepinephrine, dexamethasone, and rosiglitazone. On day 9, media was exchanged, and NDR was either readded or withdrawn. Cells were also treated with 1 μ g/ml actinomycin alone or in combination with NDR and harvested 9 h later. Results are means $\pm$ SE from 3 independent experiments performed in duplicate (normalized to 18S RNA). The value at time 0 h was set to 100%, and the curves were drawn by the "linear" function of Kaleidagraph. The *Elovl3* mRNA half-life was estimated by extrapolation of the curve to 20–25 h.

As a conclusion, our data here show that, in brown adipocytes, *Elovl3* expression is induced during induced lipolysis (thermogenesis) and adipocyte differentiation by transcriptional activation through adrenergic signaling (norepinephrine) and PPAR γ activation in combination with the exposure of glucocorticoids. As a sequential event, the elevated *Elovl3* mRNA level is maintained by norepinephrine/PPAR γ /glucocorticoid-stimulated mRNA stability (Fig. 9).

DISCUSSION

Available data favor that ELOVL activity is controlled by transcriptional regulation, which emphasizes the importance of studies involving factors regulating the mRNA levels of the respective elongase (20).

Of particular interest is the fact that the expression of *Elovl3* is elevated by >200-fold in BAT of mice exposed to a 3-day cold stress. This is qualitatively mimicked by continuous administration of norepinephrine via osmotic minipumps in mice kept at 28°C (52). However, a similar increase in expression is also found during prenatal development just before birth, i.e., before the animals experience a cold environment (52), demonstrating that *Elovl3* expression is not controlled exclusively by norepinephrine but by additional factors as well, which are highly correlated with the recruitment of BAT.

In this study, we show that norepinephrine and the PPAR γ ligand rosiglitazone together with the synthetic glucocorticoid dexamethasone induce the mRNA level of *Elovl3* in cultured brown adipocytes in a synergistic manner involving both transcriptional activation and mRNA stability.

Primary cultured brown adipocytes are the only cell culture system described to express significant amounts of *Elovl3* mRNA, and only provided that the cells are exposed to stimulatory agents. Originally, the cells were shown to require a 3-day simultaneous stimulation with both norepinephrine and dexamethasone (52). Further induction of *Elovl3* expression could be accomplished with the addition of the PPAR α ligand WY-14,643, along with induced expression of enzymes controlling fatty acid β -oxidation (19). When we, in this study, compared the capacity of the different PPAR ligands to stimulate *Elovl3* expression, the PPAR γ ligand rosiglitazone was shown to be a more potent inducer of *Elovl3* expression than the PPAR α and PPAR δ ligands. Despite this, *Elovl3* mRNA levels have been shown to be significantly reduced in PPAR α -ablated mice, both in BAT and in cultures of brown adipocytes (19), implying that, although the potency of the PPAR α ligand was relatively low, the significance of its role in stimulating fatty acid synthesis in BAT via *Elovl3* expression is indisputable. Whether PPAR δ is involved in the regulation of *Elovl3* expression in BAT is still unclear. Because L-165041 works as a PPAR δ agonist within the nanometer range and has been shown to have significant PPAR γ agonist activity in the micrometer range (14, 29), it may suggest that the effect of L-165041 in our study is via PPAR γ .

That *Elovl3* expression can be induced by different PPAR ligands is not a unique feature. All three PPAR subtypes have been reported to be coexpressed in differentiated brown adipocytes (26, 32, 54). The BAT-specific UCP1 has, for example, been found to be regulated by both PPAR α (3) and PPAR γ (44, 46). The UCP1 promoter contains a PPAR-responsive element (PPRE) that behaves as a promiscuous responsive site to either PPAR α or PPAR γ activation but not to PPAR δ (3).

Fig. 8. Cycloheximide reduces *Elovl3* mRNA stability. Primary cultures of brown adipocytes were cultured for 9 days. The last 3 days in culture, cells were treated with norepinephrine, dexamethasone, and rosiglitazone. Before harvest (9 h), 50 μ M cycloheximide or 1 μ g/ml actinomycin was added. Results are means $\pm$ SE from 3 independent experiments performed in duplicate (normalized to 18S RNA). The value at time 0 h was set to 100%.

Fig. 9. Tentative scheme for mediation of the effect of norepinephrine (NE), rosiglitazone, and dexamethasone on the expression of *Elovl3* in brown adipocytes during induced lipolysis (thermogenesis) and/or adipocyte differentiation. Norepinephrine, rosiglitazone, and dexamethasone are stimulating *Elovl3* expression via adrenergic receptors (AR), PPAR γ , and glucocorticoid receptor (GR), respectively, both on the level of transcription as well as increased mRNA stability through a mechanism that requires novel protein synthesis.

Correspondingly, the *Elovl3* promoter contains a putative PPAR/retinoid X receptor binding site ~400 bp upstream of the transcription start site; whether this is a functional site for any PPAR isoform has to be confirmed in the future.

In this study, we show that *Elovl3* expression is tightly connected to the differentiation status of the cells and that the introduction of rosiglitazone in addition to norepinephrine-dexamethasone stimulation results in a synergistic enhancement of *Elovl3* expression, suggesting that the intrinsic differentiation program of the cells, i.e., induced lipid accumulation, is enhanced by the induced *Elovl3* expression.

Because *Elovl3* mRNA can be induced by both PPAR α (19) and PPAR γ activation, we propose two different, but highly coordinated, metabolic pathways regulating *Elovl3* expression. One, which is associated with the induction of the fatty acid oxidation machinery mediated via PPAR α , and another pathway, which is linked to adipogenic differentiation (i.e., fat accumulation and mitochondriogenesis), are mediated via PPAR γ . The activity can then be further stimulated by the adrenergic pathway as fatty acid oxidation is induced, and, accordingly, an increased demand for fatty acid supplementation is required (2, 6, 8). However, it is clear from our data that these pathways require glucocorticoid input to optimally activate *Elovl3* expression. Interestingly, as we show here, in combination with dexamethasone, rosiglitazone is a more potent stimulator of *Elovl3* expression than norepinephrine, indicating that induced differentiation has a stronger impact on *Elovl3* expression than sympathetic activation of thermogenesis, i.e., induced fatty acid oxidation. At least, the rosiglita-

zone-dexamethasone signaling could explain the induced *Elovl3* expression seen in fetal animals just before birth when the animals have not yet experienced any cold stimuli (52). This is also the point when PPAR γ appears in BAT during embryogenesis (7). In addition, the reduced amount of fat droplets seen in *Elovl3*-ablated mice at thermoneutral conditions (30°C) support the existence of a nonadrenergic control of *Elovl3* and lipid mobilization in brown adipocytes, even before the animals have experienced any cold stimuli (57).

The mechanism behind the glucocorticoid-induced *Elovl3* expression is not known. In adipocyte cell lines, dexamethasone alone is unable to induce adipocyte differentiation. However, in a rather complex manner, glucocorticoids, in combination with increased cAMP or supply of exogenous PPAR γ ligands, can control the transcriptional activity and conversion into mature adipocytes (28). Administration of norepinephrine and rosiglitazone together resulted in a synergistic increase of *Elovl3* expression within 6 h of treatment, reaching maximal levels after 1 day. The time frame of induction is very similar to what is seen in vivo when mice are placed in a cold environment.

Rosiglitazone-induced gene expression has been reported to give maximal mRNA levels within 24 h, both for UCP1 and the adipose-specific fatty acid-binding protein aP2/FABP4 (35, 42, 50). In fetal rat brown adipocytes, rosiglitazone has been shown to induce UCP1 expression significantly after 1 h; however, a fourfold induction required a 24-h treatment (48).

Norepinephrine-induced gene expression is usually more rapid. For example, UCP1 mRNA levels start to increase within 15 min of norepinephrine treatment (38, 39) and reach maximal levels within 8 h (17). Norepinephrine-induced *Elovl3* expression is less rapid, with a detected increase after 6 h that peaks after 24 h of treatment (this study). However, although norepinephrine and rosiglitazone were able to stimulate *Elovl3* expression up to maximal mRNA levels within 24 h, the cells required preexposure to dexamethasone for ~2–3 days to do so.

Transcriptional regulation has been shown to be important for several enzymes involved in fatty acid synthesis. One of the most studied fatty acid synthesizing enzymes, FAS, has been shown to be regulated only on the transcriptional level (34). The fact that *Elovl3* gene expression is induced heavily upon cold exposure in mice implies that transcriptional regulation of *Elovl3* is important. Indeed, our data here on brown fat primary cultures imply that induction of *Elovl3* mRNA levels by both norepinephrine and rosiglitazone occurred through increased transcription, since the induction was inhibited by actinomycin treatment. Furthermore, because the addition of the protein synthesis inhibitor cycloheximide totally abolished both norepinephrine- and rosiglitazone-induced expression, it suggests that the induced transcription is achieved via a signaling cascade involving novel protein synthesis. In accordance with this, to obtain full effect by norepinephrine within an 8-h period, it was sufficient to expose the cells to NE only during the first hour of the experiment (data not shown).

Concerning an eventual posttranscriptional regulation, the half-life of *Elovl3* mRNA was shown to differ depending on whether the cells were exposed to the stimulatory mix or not. Furthermore, in the presence of the protein synthesis inhibitor, the half-life was even shorter, ~9 h, implying that cycloheximide, in addition to blocking *Elovl3* transcription, also inhibits the synthesis of a protein stabilizing the *Elovl3* mRNA. Be-

cause withdrawal of the norepinephrine-dexamethasone-rosiglitazone mixture, but not inhibition of transcription, reduced the *Elovl3* mRNA level, it suggests that *Elovl3* mRNA degradation is prevented in the presence of norepinephrine-dexamethasone-rosiglitazone. However, from our data, it is not clear whether all three components are necessary for this activity. The reduced mRNA level seen in Figs. 5 and 8 after cycloheximide treatment may suggest that dexamethasone, as the common denominator in these experiments, is required for enhanced *Elovl3* mRNA stability.

In conclusion, our data suggest that norepinephrine and the PPAR γ ligand rosiglitazone induce *Elovl3* mRNA levels in the brown adipocyte through a series of events involving stimulation of transcription and stabilization of the mRNA, which require novel protein synthesis. Because the time frame of which both norepinephrine and rosiglitazone induce *Elovl3* expression is very similar, one scenario could be that the norepinephrine-induced *Elovl3* expression is due to the release of certain fatty acids, which may act as PPAR γ ligands. However, because the combination of norepinephrine and rosiglitazone induces *Elovl3* expression in a highly synergistic manner, it implies two separate pathways with the aim to synthesize very long chain fatty acids for triglyceride and lipid droplet formation.

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