Control of 4E-BP1 expression in mouse brown adipose tissue by the β_3-adrenoceptor

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Abstract Knockout of the translation inhibitor 4E-BP1 induces an overexpression of uncoupling protein-1 (UCP1) [Nature Medicine 7 (2001) 1128]. A possible inverse control of UCP1 and 4E-BP1 expressions in mouse brown adipose tissue was investigated. Cold-exposure, which increases the expression of UCP1, decreased that of 4E-BP1 mRNA in wild type but not in $\beta_1/\beta_2/\beta_3$-adrenoceptor knockout mice. Administration of the β_3-adrenoceptor agonist CL 316 246 decreased 4E-BP1 mRNA by 75% and protein by 41% after 6 and 48 h, respectively. Our data are the first report of a regulation by the β_3-adrenoceptor of 4E-BP1 expression. They support a role of the latter in adaptive thermogenesis.

Keywords: 4E-BP1 expression; UCP1; β-Adrenoceptor; Brown adipose tissue

1. Introduction

Brown adipose tissue (BAT), which is involved in the control of body temperature and body weight via non-shivering and diet-induced thermogenesis, plays an important role in mammalian energy balance. It contains a unique mitochondrial uncoupling protein, termed uncoupling protein-1 (UCP1), which produces heat by uncoupling oxidative phosphorylation. BAT also expresses the peroxisome proliferator-activated receptor γ (PPARγ) co-activator-1 (PGC-1), sharing this feature with the heart and skeletal muscle of cold-exposed rodents. PGC-1 stimulates the transcriptional activity of PPARγ and thyroid hormone receptors on UCP1 expression in BAT [2]. Cold-exposure results in an activation of the BAT sympathetic nervous system (SNS), which stimulates, via the β-adrenoceptors, PGC-1 and UCP-1 expression [2].

The initiation phase of mRNA translation is a key step in the control of protein synthesis. It involves the recognition of the mRNA 5′ cap structure by the eukaryotic initiation factor 4F (eIF4F) cap-binding complex. This complex consists of 3 subunits: eIF4A, eIF4E and eIF4G [3]. The activity of eIF4E is regulated by its interaction with a family of three inhibitory proteins, the eIF4E binding proteins (4E-BPs). In its underphosphorylated form, 4E-BP1 binds to eIF4E and prevents the formation of the eIF4F complex, thus inhibiting cap-dependent translation. 4E-BP1 hyper-phosphorylation causes its dissociation from eIF4E, which can form a productive eIF4F complex [4,5]. It has been shown that the anabolic effects of hormones, like insulin and growth hormone, or of nutrients implicate the phosphorylation of 4E-BP1 in various tissues [6,7].

4E-BP1 knockout (4E-BP1 KO) mice were recently reported. They were expected to display a global increase in tissue protein synthesis. The phenotype of the 4E-BP1 KO mice was, however, a surprise. The inguinal and retro-peritoneal white adipose tissues (WAT) of 4E-BP1 KO mice were found to contain an increased number of multilocular adipocytes, which are characteristic of BAT. Consistent with this, WAT PGC-1 protein and UCP1 mRNA levels, as well as whole body energy expenditure, were increased, resulting in a decrease in body fat mass. 4E-BP1 therefore seems to be a specific repressor of UCP1 expression and can be considered a new player in the control of adipose tissue development and energy homeostasis [1].

Since the activation of the BAT SNS by cold-exposure results in an increase in the expression of the thermogenic partners PGC-1 and UCP1 [2], it was hypothesized that the BAT SNS also acts on 4E-BP1 expression in a way that would facilitate the increase in thermogenesis. The aim of the present study was therefore to investigate the possible downregulation of the mouse BAT 4E-BP1 mRNA and protein expressions by the β-adrenergic system.

2. Materials and methods

2.1. Mice

Animals were treated in accordance with our institutional guidelines. Three month-old female C57BL/6J wild type (WT) or β_3-adrenoceptor KO mice generated in our laboratory [8] were used. The β_1/β_2-adrenoceptor KO mice [9] were kindly provided by Dr. B.K.

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Abbreviations: BAT, brown adipose tissue; UCP1, uncoupling protein-1; PPARγ, peroxisome proliferator-activated receptor γ; PGC-1, PPARγ co-activator-1; eIF4F, eukaryotic initiation factor 4F; 4E-BP, eIF4E binding protein; KO, knockout; WAT, white adipose tissue; WT, wild type; SNS, sympathetic nervous system; PKA, protein kinase A; MAPK, mitogen-activated protein kinase

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Kobilka (Howard Hughes Medical Institute, Stanford, CA, USA). The $\beta_1/\beta_2/\beta_3$-adrenoceptor KO (β-less) mice were generated in our laboratory [10] by intercrossing our β_3-adrenoceptor KO [8] with the β_1/β_2-adrenoceptor KO mice [9]. WT and KO colonies were on the same mixed genetic background (129SvJ, FVB/N, C57BL/6J and DBA/2 for β_1/β_2-adrenoceptor KO mice and the same plus 129 Sv/ev for β-less mice). All animals were maintained at 24 °C on a 12 h/12 h light/dark cycle (7:00–19:00), with free access to water and standard laboratory chow diet (Nordos, Cergy, France) and caged individually during the experimental periods. In one experiment, C57BL/6J mice were injected with saline or CL 316243 (1 μg/g of body weight) intra-peritoneally.

2.2. Northern blots

The mice were killed by cervical dislocation and the interscapular BAT was carefully dissected and quickly frozen in liquid nitrogen. Total RNA was purified by the method of Chomczynski and Sacchi [11], and 15 μg of RNA was electrophoresed in 1% agarose gel containing formaldehyde and transferred according to standard protocols. The probes used were full length mouse UCP1 cDNA (GenBank Accession No. U63418) and a 701 bp 4E-BP1 PCR probe, position 22–722 (GenBank Accession No. NM007918). The probes were labeled by random priming with [α-^{32}P] dCTP (Amersham International, Bucks, UK) to a specific activity of approximately 1×10^9 d.p.m./μg DNA. Hybridizations were performed using Quickhyb™ solution (Stratagene Inc, La Jolla, CA) as previously described [12]. Subsequent hybridization of the blots with a [α-^{32}P] ATP labeled synthetic oligonucleotide specific for the 18S rRNA subunit was used to correct for the differences in the amounts of RNA loaded onto the gels. Blots were exposed at −80 °C to Hyperfilm ECL films (Amersham International, Bucks, UK). RNA levels were quantified by scanning photodensitometry of the autoradiograms using IMAGEQUANT Software version 3.3 of Molecular Dynamics (Sunnyvale, CA).

2.3. Real-time PCR

Oligo-dT first strand cDNA were synthesized using the Superscript™ II RNase H Reverse Transcription kit (Invitrogen™, Life technologies, Basel, Switzerland) according to the manufacturer's instructions. Real-time PCR was performed using ABI rapid thermal cycler system and a SYBR Green PCR master mix according to the manufacturer's instructions. Cyclophilin A was used as a control to account for any variations due to the efficiencies of the reverse transcription and PCR. UCP1 oligonucleotide primers used were: upstream 5′-cgatgtccatgtacaccaagga-3′ and downstream 5′-ttgtggcttcttttctgcga-3′, covering the nucleotides 996–1063 of UCP1 cDNA (GenBank Accession No. U63418). 4E-BP1 oligonucleotide primers used were: upstream 5′-ggcggcacgctcttca-3′ and downstream 5′-gaaattttctgatggagtgtcgga-3′, covering the nucleotides 120–195 of 4E-BP1 cDNA (GenBank Accession No. NM007918). Cyclophilin A oligonucleotide primers used were: upstream 5′-caaatgctggaccaaacacaa-3′ and downstream 5′-ccatccagccattcagtcttg-3′, covering the nucleotides 343–412 of cyclophilin A (GenBank Accession No. XM355936). The conditions of PCR were a step at 50 °C for 2 min followed by a denaturing step at 95 °C for 10 min and by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The upstream and downstream oligonucleotide primers were chosen on both sides of an intron to prevent amplification of possible contaminating genomic DNA.

2.4. Western blots

BAT were minced with scissors in 10 volumes of an ice-cold homogenization buffer containing 25 mM sucrose, 12.5 mM Tris/HCl, pH 7.4, and 0.06% protease inhibitor cocktail (Sigma, St Louis, MO). They were then homogenized using a Teflon pestle in an ice-cold Potter–Elvehjem homogenizer (clearance 0.11 mm, 10 up and down strokes, 1800 r.p.m.). The homogenate was centrifuged at $1600 \times g$ for 10 min and the supernatant filtered through 1 layer of surgical gauze and centrifuged again at $35\,000 \times g$ for 30 min. The final supernatant was kept for Western blot analysis. The protein concentration was measured as described by Bradford [13] using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA), with BSA as a standard. Thirty micrograms of supernatant was dried under vacuum and resuspended in 10 μl of a loading buffer containing 20% glycerol, 6% SDS and 0.02% bromophenol blue in 0.25 M Tris/HCl, pH 6.8. Western blots were performed as previously described [14] using rabbit polyclonal antibody #11211, which detects mouse 4E-BP1 and 4E-BP2 [1] and a mouse monoclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (Chemicon International, Temacula, CA). The signals were quantified by scanning photodensitometry as described above.

2.5. Blood parameters

RIA kits were used for free T3 (ICN Pharmaceuticals, Orangeburg, NY) and glucocorticoids (IDS, Boldon, UK) measurements.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Significance was evaluated using the unpaired Student's t test.

3. Results and discussion

The effects of cold-exposure on UCP1 expression are mediated by an activation of the SNS acting via the β-adrenoceptor signaling system [2]. The degrees of expression of UCP1 and 4E-BP1 mRNA were first compared in the BAT of WT mice maintained at 24 °C or exposed to 6 °C for 48 h. As shown in Fig. 1, cold-exposure increased UCP1 mRNA expressions 1.8-fold, but decreased 4E-BP1 mRNA expression by 38% in the BAT. Thus, cold-exposure induces an inverse modulation of UCP1 and 4E-BP1 mRNA expressions in the BAT. These data are the first report of a regulation of 4E-BP1 mRNA expression in vivo. A regulation of 4E-BP1 expression has until now been reported only in hematopoietic cell lines [15]. The observed inhibition of 4E-BP1 expression reported in this study could play a role in the upregulation of UCP1 upon cold-exposure in mouse BAT.

Cold-exposure has been shown to induce the emergence of brown adipocytes and an increase in UCP1 expression in the WAT [16]. In fact, exposure to 6 °C for 48 h induced a 2.6-fold increase in UCP1 mRNA expression in the mouse parametrial WAT [17]. This same treatment did not change 4E-BP1 expression in the parametrial WAT (100 ± 5% vs 97 ± 7% in control as compared to 48 h cold-exposed mouse WAT,

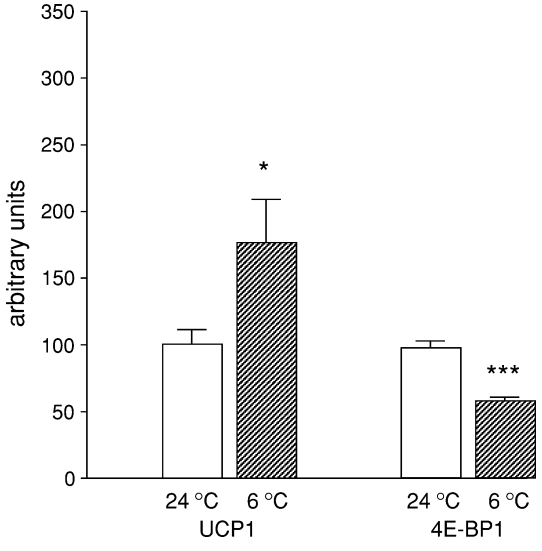

Fig. 1. Northern blot quantification of UCP1 and 4E-BP1 mRNA in the BAT of C57BL/6J female mice maintained at 24 °C (controls) or exposed to 6 °C for 48 h. The results are means ± S.E. of arbitrary values normalized using the corresponding 18S rRNA values. They are expressed as percent of the controls. $n = 6$; $^*P < 0.05$ and $^{***}P < 0.005$ vs respective control values.

respectively; $n = 6$). This result confirms the hypothesis made previously that the brown adipocytes present in the WAT differ from those in the BAT [17].

To test if the effect of cold-exposure on 4E-BP1 mRNA expression is, like that on UCP1, mediated by the β-adrenergic signaling, we used the β-less mouse model generated in our laboratory [10]. In the WT mouse BAT, UCP1 protein expression was increased 1.9-fold, whereas in the β-less mouse BAT it was not significantly modified after 48 h of cold-exposure [10]. As shown in Fig. 2, 4E-BP1 mRNA expression was similar in the BAT of WT and β-less mice. Cold-exposure decreased 4E-BP1 mRNA expression by 45%, 60%, and 48% after 2, 6 and 12 h, respectively. These results show that the inhibition by cold-exposure of BAT 4E-BP1 expression is a general phenomenon occurring in different genetic backgrounds. They also show that this inhibition is rapid, being large and significant already after 2 h at 6 °C. In the BAT of β-less mice, 4E-BP1 mRNA expression tended to increase reaching a significant value of 1.7-fold after 6 h of cold-exposure. The suppression of the cold-exposure effect in β-less mouse BAT suggests that the inhibition of 4E-BP1 expression is under the control of the SNS. The unexpected increase of 4E-BP1 expression after 6 h of cold-exposure suggests the existence of other modulator(s) of 4E-BP1 expression.

The effects of catecholamines on UCP1 expression are known to be mediated in mouse BAT by the three β-adrenoceptor subtypes, the β_3-adrenoceptor being predominant [18]. To determine the respective participation of the β_1/β_2- and of the β_3-adrenoceptor in the downregulation of 4E-BP1 expression in BAT, we used β_1/β_2- and β_3-adrenoceptor KO models. As shown in Fig. 3, in the BAT of the β_1/β_2-adrenoceptor KO mice, 4E-BP1 mRNA expression was decreased by 34% whereas, in the BAT of the β_3-adrenoceptor KO mice, it was increased 1.3-fold as compared to the respective WT controls. These results suggest that the inhibitory effect of the β-adrenergic signaling on 4E-BP1 mRNA expression in the BAT is not mediated by the β_1/β_2, but essentially by the β_3-adrenoceptor.

To confirm the possibility that the downregulation of 4E-BP1 mRNA expression is mediated by the β_3-adrenoceptor, we tested the effect of the β_3-adrenoceptor agonist CL 316 243 in vivo. The expression levels of UCP1 mRNA and of 4E-BP1 mRNA and protein in the BAT of CL 316 243 and saline injected mice were measured. The administration of the β_3-adrenoceptor agonist BRL 26830A has been shown to induce a rapid and transient increase in UCP1 mRNA expression in BAT [19]. Similarly, as shown in Fig. 4, UCP1 mRNA levels were increased 1.8-fold after 6 h and returned to near control value 12 h after the injection of CL 316 243. 4E-BP1 mRNA

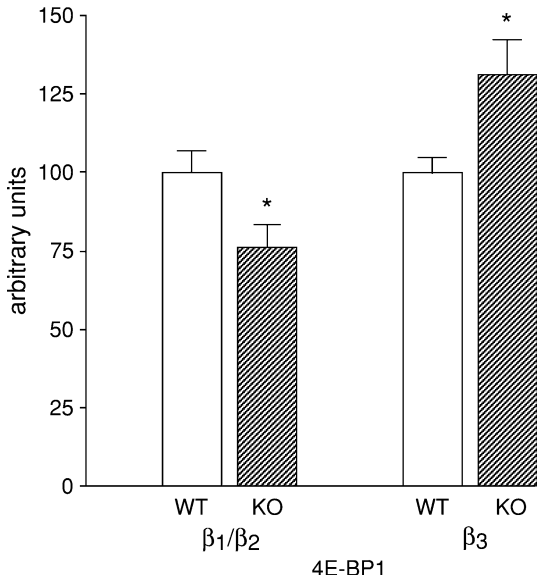

Fig. 3. Northern blot quantification of 4E-BP1 mRNA in the BAT of WT, β_1/β_2-, or β_3-adrenoceptor KO mice. The results are means ± S.E. of arbitrary values normalized using the corresponding 18S rRNA values. They are expressed as percent of the controls. $n = 6$; $^*P < 0.05$ vs respective control values.

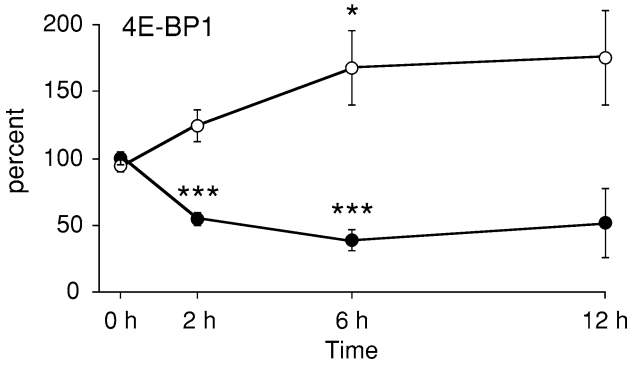

Fig. 2. Real time PCR quantification of 4E-BP1 mRNA in the BAT of WT (●) and β-less (○) female mice exposed to 6 °C for 2, 6 and 12 h. The results are means ± S.E. of arbitrary values normalized using the corresponding cyclophilin A values. They are expressed as percent of the values at time 0 in the WT mouse BAT. $n = 3$–5; $^*P < 0.05$ and $^{***}P < 0.005$ vs time 0 values.

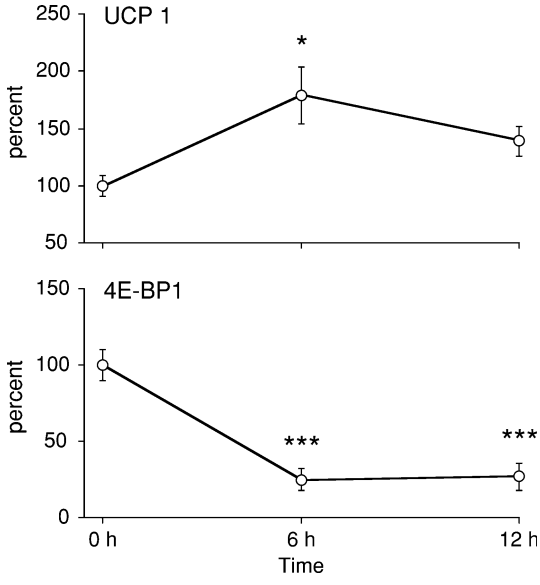

Fig. 4. Real time PCR quantification of UCP1 and 4E-BP1 mRNA in the BAT of C57BL/6J female mice 6 or 12 h after the intraperitoneal injection of 1 μg/g of body weight of CL 316 243. The results are means ± S.E. of arbitrary values normalized using the corresponding cyclophilin A values. They are expressed as percent of the values obtained in the saline injected controls. $n = 6$; $^*P < 0.05$ and $^{***}P < 0.005$ vs control values.

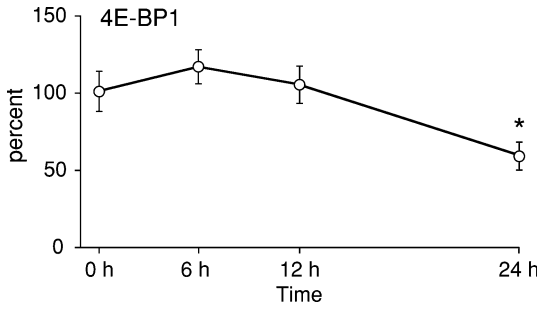

Fig. 5. Western blot quantification of 4E-BP1 protein in the BAT soluble fraction of C57BL/6J female mice 6, 12 or 24 h after the intraperitoneal injection of 1 μg/g of body weight of CL 316 243. The results are means ± S.E. of arbitrary values normalized using the corresponding glyceraldehyde phosphate dehydrogenase (GAPDH) values. They are expressed as percent of the values observed in saline injected controls. $n = 3$; $^*P < 0.05$ vs time 12 h value.

levels were decreased by 75% and 73% 6 and 12 h after the injection, respectively. The β_3-adrenoceptor seems therefore to be largely involved in the control of 4E-BP1 expression by cold-exposure. The β_3-adrenergic stimulation of UCP1 transcription is mediated by cyclic AMP and protein kinase A (PKA) in a p38 mitogen-activated protein kinase (MAPK)-dependent or independent manner [20]. It would be interesting to know which PKA downstream component of the signaling cascade participates in the downregulation of 4E-BP1 mRNA expression.

We also measured the effect of CL 316 243 in vivo on 4E-BP1 protein expression. As shown in Fig. 5, the level of 4E-BP1 protein was maintained up to 12 h after CL 316 243 injection, but fell by 41% 24 h after the injection. The decrease in 4E-BP1 protein expression is probably not involved in the acute control of UCP1 expression by CL 316 243. It might however play a role in the maintenance of an increased UCP1 expression upon the prolonged β_3-adrenoceptor stimulation occurring during cold-acclimation. It would be interesting to investigate if 4E-BP1 phosphorylation is modulated by the SNS and plays a role in the acute control of UCP1 expression in the BAT.

The observation of a control of BAT 4E-BP1 expression by catecholamines suggests that other hormones, involved in the control of BAT thermogenesis, might participate in the modulation of 4E-BP1 mRNA expression. Interesting candidates might be the thyroid hormone T3 and glucocorticoids. In BAT cells, T3 has been shown to synergize with norepinephrine to increase UCP1 expression [21] and glucocorticoids to inhibit the effect of norepinephrine on UCP1 expression [22]. In β-less mice maintained at 24 °C, the blood levels of T3 and of glucocorticoids were found to be 1.3- and 1.6-fold higher, respectively, than in WT controls ($P < 0.05$, $n = 5$; results not shown). Despite these increases, 4E-BP1 mRNA expression was not modified in the BAT of β-less as compared to WT mice at 24 °C (Fig. 2). This observation does not rule out the possibility that T3 and glucocorticoids might play a role in the control of 4E-BP1 expression upon cold-exposure.

The present study is the first to describe a control of 4E-BP1 expression by the SNS. This control is in line with a possible role of 4E-BP1 in the complex mechanism of the β_3-adrenoceptor-mediated increase in BAT UCP1 expression.

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