The TRP channel agonists nonivamide and cinnamaldehyde augment cold-induced mitochondrial biogenesis in white adipocytes

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Abstract

An increased conversion of white adipocytes to a "brite" adipocyte phenotype, characterized by increased number of mitochondria and increased expression of uncoupling protein 1 (UPC-1), represents a promising anti-obesity approach. Beside an activation of beta-adrenergic signalling by a cold ambient temperature, also the activation of several temperature-sensing transient receptor potential (TRP) ion channels has been associated with browning of white adipocytes. In the present study, we hypothesized that the two food-derived aroma compounds and TRP-channel agonists nonivamide and cinnamaldehyde augment the thermogenic response of 3T3-L1 white adipocytes to a cold stimulus. We found that upon incubation at 29°C, 3T3-L1 adipocytes show an increased expression of UCP-1 at the levels of gene transcripts and protein level as well as increased mitochondrial biogenesis in comparison to 37°C, confirming the validity of the cellular model. In addition, we demonstrate here that treatment of 3T3-L1 cells with 0.1 µM nonivamide or 10 µM cinnamaldehyde for 48 h increased mitochondrial biogenesis when incubated at 29°C, but not at 37°C. These data hint towards beneficial effects of the two aroma compounds when applied as a chronic treatment in addition to a cold stimulus, which has to be validated in future studies.

Introduction

For the first time, the current generation of humans may have a shorter life span than the previous [1]. This is mostly due to an inactive lifestyle combined with the consumption of energy-dense food, resulting in an expanding population of chronically ill people with obesity-associated diseases [2]. This underlines the urgent need for new weight loss-supporting measures.

Weight loss is achieved by a negative energy balance, which can be accomplished by an increased energy expenditure, determined by the total metabolic rate. The total metabolic rate is divided into basal metabolic rate, physical activity, and thermogenesis [3]. Recent calculations demonstrate that the adaptive thermogenesis, which is mediated by brown adipose tissue, is accounting for ~5% of the total thermogenesis and can be a relevant target to achieve a difference in body weight [4]. The brown adipocytes are, in contrast to white adipocytes, densely packed with mitochondria that express UCP-1. Upon activation, this protein shortcuts the circuits of the electrochemical gradient of the respiratory chain, which is the driving force in ATP production. Thus, UCP-1 activates heat production from available substrates, leading to an increased energy expenditure [5]. More recently, upon stimulation, clusters of UCP-1 expressing adipocytes with an increased number of mitochondria have been identified in white adipose tissue. This specific phenotype of cells is often referred to as "brite" (brown in white) adipocytes [6, 7].

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The probably most widely studied inducer of a brite adipocyte phenotype is cold temperature, which stimulates sympathetic nerve activity by beta-adrenergic receptor activation, followed by increased cyclic adenosine monophosphate (cAMP) and protein kinase A levels, finally leading to activation of UCP-1 [8].

Beside a cold ambient temperature, also activation of the warm-temperature-sensing transient receptor potential (TRP) ion channels TRPV1 [9] and TRPV4 [10] has been associated with browning of white adipocytes. In addition, also the food-derived TRPA1 agonist cinnamaldehyde has been demonstrated to unfold thermogenic effects in primary white adipocytes [11]. However, the response of TRP channel agonists in addition to cold ambient temperatures has not been studied so far. Thus, in the present study, we hypothesized that the food-derived aroma compounds nonivamide and cinnamaldehyde, which are agonists for the TRPV1 or TRPA1 channel, respectively, may augment cold-induced browning-responses in 3T3-L1 white adipocytes as a model. The first part of this study investigated the suitability of the 3T3-L1 cells to demonstrate browning of white adipocytes using UCP-1 gene expression and protein level, and mitochondrial biogenesis as novel marker for compound screening. The second part of the study assessed the effects of the TRPV1 agonist nonivamide and the TRPA1 agonist cinnamaldehyde on mitochondrial biogenesis with or without an additional cold stimulus.

Experimental

Materials

3T3-L1 (mouse fibroblasts) cells were purchased at ATCC. Nonivamid was kindly provided by Symrise AG, Holzminden, Germany. All other used reagents were obtained from Sigma Aldrich, Austria, unless stated otherwise.

Cell culture

3T3-L1 pre-adipocytes were cultured and differentiated to mature adipocytes as described before [12]. Adipocytes were used for experiments on day 10 after initiation of differentiation. Incubation of the cells was carried out in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 4.5 [g/L] glucose and 2 mM pyruvate in the absence of phenol red and bicarbonate.

Cell viability

Effect of applied concentrations of cinnamaldehyde (CA, 10-100µM) and nonivamide (NV, 0.1-1µM) on cellular proliferation as a measure for the viability of the cells was analysed via standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Carl Roth, Germany) assays as described before [12].

Quantitative real time-polymerase chain reaction

UCP-1 gene expression was analysed in mature 3T3-L1 cells by using quantitative Real-Time PCR. The total RNA was isolated using MasterPure™ RNA Purification Kit (Epicentre®) according to manufacturer's protocol. Quality and concentration of isolated RNA was analysed using NanoQuant Plate on an Infinite M200 Plate reader (both Tecan). RNA was subsequently reversely transcribed into cDNA using the high capacity RNA to cDNA Kit (ABI, Thermo Fisher) and the PCR was performed on a StepOnePlus device (ABI, Thermo Fisher) using SYBR Green Fast Master Mix (ABI, Thermo Fisher). Gene expression of *UCP-1* was measured in triplicates and normalized to the reference genes *HPRT* and *ACTB* as endogenous controls. The hypothetical respective starting mRNA levels were calculated using LinReg v 12.8 software and presented as fold change relative to control cells (37°C, set to 1). Detailed Primer information can be obtained in Table 1.

Table 1: Sequences of the primers used during PCR reaction.

Gene	*Sequence (3'-5')*	*Product length [bp]*	*Reference*
HPRT	FW: GAGAGCGTTGGGCTTACCTC RV: ATCGCTAATCACGACGCTGG	136	[12]
ACTB	FW: TCTTTGCAGCTCCTTCGTTG RV: CATTCCCACCATCACACCCT	188	[13]
UCP-1	FW: AGGCTTCCAGTACCATTAGGT RV: CTGAGTGAGGCAAAGCTGATTT	133	[14]

Mitochondrial biogenesis

For assessing mitochondrial biogenesis, mitochondria were stained using MitoTracker® Deep Red (Molecular Probes, Thermo Fisher) after incubation of 3T3-L1 adipocytes at 37°C or 29°C for 48 h in a 96-well format with or without addition of the test substances based on methods described by Chowanadisai et al. [15] and Huang et al. [16]. In brief, cells were incubated with 50 nM MitoTracker ® dissolved in phenol red-free DMEM for 30 min, and washed three times with phenol red-free DMEM before fluorescence was measured at 640 nm excitation and 665 nm emission by means of a Tecan Infinite M200 (Tecan) plate reader. Results were calculated in percent relative to untreated control cells.

Immunocytochemistry

For intracellular UCP-1 staining, 3T3-L1 pre-adipocytes were differentiated on round glass slides (12mm, Carl Roth) in 24-well plates (Sarstedt) before incubation at 37°C or 29°C for 48 h. After fixation with 4 % formalin and blocking for 60 min with 5% FBS (Gibco) and 0.5% Trition-X100 (Carl Roth), cells were incubated under gentle agitation with a specific UCP-1 primary antibody (1:200; Abcam) for further 60 min. Detection was carried out using Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes). The corrected total cell fluorescence (CTFC) was analysed using Image J 2.0.0. As a control, background fluorescence intensity after incubation of the cells with the secondary antibody alone was analysed (data not shown).

Statistical analysis

Data are presented as fold change or percent of control ± SEM, calculated from at least three different experiments with multiple technical replicates each after excluding outliers identified using Nalimov outlier test. Normal distribution and equal variance of the data were tested using Shapiro-Wilk or Brown-Forsythe test, respectively. Significant differences between two groups were assessed using T-Test, or Mann-Whitney-U test, in case of not normally distributed data. Likewise, significant differences between multiple treatments were tested using One-Way ANOVA with Holm-Sidak post hoc test, or One-Way ANOVA on Ranks with Dunn's post-hoc test, respectively. Differences between treatment groups are marked with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. SigmaPlot 13.0 was used for statistical analysis.

Results and discussion

Browning of white adipocytes is, amongst others, characterized by an increased number of mitochondria combined with an upregulation of *UCP-1* expression [17]. The

process of browning can not only be mediated by cold-stimuli via beta-adrenergic signalling, but also by activation of TRP channels like TRPV1 and TRPV4 [9, 10]. In the present study, we hypothesized that the food-derived aroma compounds nonivamide (NV) and cinnamaldehyde (CA), which are agonists for TRPV1 or TRPA1 cation channels, respectively, may augment cold-induced browning responses in 3T3-L1 white adipocytes.

In a first set of experiments, 3T3-L1 adipocytes were tested for their browning response when exposed to a cold ambient temperature. Since pyruvate serves as an easily catabolized substrate for mitochondrial futile cycles, and pyruvate metabolism partly regulates lipogenesis during cold exposure [18], the impact of pyruvate on *UCP-1* expression was addressed as well. *UCP-1* gene expression was analysed as a marker of the induction of a beige phenotype in 3T3-L1 cells after exposure to 29°C for 6 h (Figure 1A), and revealed an 8.98 ± 3.61 fold increased expression. When no pyruvate was added to the incubation media, the *UCP-1* gene expression was significantly reduced to a 2.06 ± 0.25 fold change increase (data not shown). This result strengthens the assumption that the increased *UCP-1* gene expression indicates a thermogenic response in 3T3-L1 adipocytes.

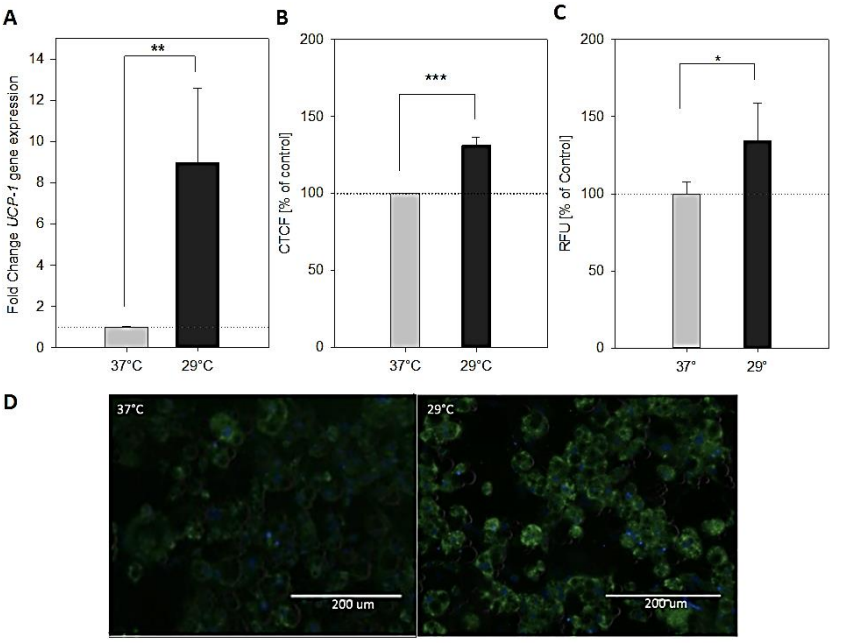

Figure 1: Analysis of *UCP-1* gene expression (**A**, n=10), UCP-1 immunostaining (**B**,**D**, analysis of the corrected total cellular fluorescence (CTCF) in a total of 50-100 cells) and mitochondrial biogenesis (**C**, n=4) in 3T3-L1 adipocytes after incubation for 6 h (**A**) or 48 h (**B**-**D**) at 37°C or 29°C. Statistics: Student's T-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

Moreover, the increased *UCP-1* gene expression was confirmed by immunocytostaining on protein level after incubation for 48 h at 29°C, demonstrating 31.3 ± 5.22% increased UCP-1 levels in cells kept at 29°C compared to cells incubated at 37°C (Figure 1B & 1D). In addition, staining of the mitochondria using a specific mitochondric dye demonstrated that the number of mitochondria was increased by 36.6 ± 24.1% after 48 h at 29°C in comparison to 37°C (Figure 1C). These data point to an

increased mitochondrial biogenesis following exposure to cold temperatures and confirm the validity of the 3T3-L1 cells as a model for browning.

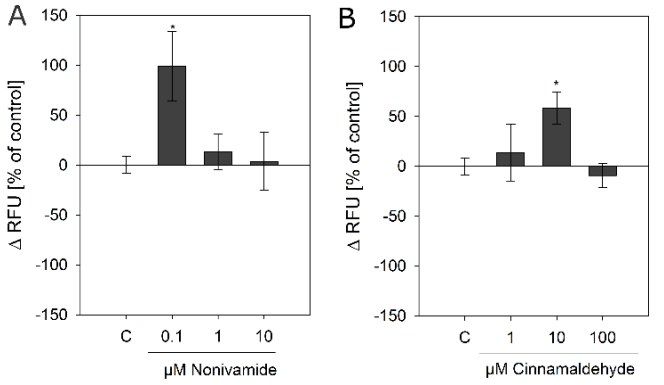

Figure 2: Differences in the mitochondrial biogenesis after treatment with 0.1-10 µM nonivamide (A) or 1 to 100 µM cinnamaldehyde (B) for 48 h at 29°C. n=3-4. Statistics: One-Way ANOVA *vs.* control with Holm-Sidak post hoc test (A), One-Way ANOVA on Ranks *vs.* control with Dunn's post hoc test (B). * $P<0.05$

Beside cold temperatures, also activation of thermosensitive TRP channels like TRPV4 and TRPV1 has been shown to induce a brite adipocyte phenotype [9, 10]. In addition, the thermosensitive TRPV1 and, more recently, also TRPA1 has been shown to be involved in the differentiation and maturation process of 3T3-L1 adipocytes [12]. Moreover, the TRPV1 agonist NV has been associated with a decreased body fat content following oral administration of 0.15 mg per day in a 12-week human intervention trial with overweight test subjects [19]. Likewise, also addition of 1% of the TRPA1 agonist CA to a high-fat diet has been associated with anti-obesity effects accompanied by increased mitochondria protein levels in mice [20]. However, it has not been clarified yet if thermogenic responses of adipocytes to a cold-stimulus can be enhanced by a treatment with NV or CA. Thus, in the present study, we investigated whether NV or CA induce mitochondrial biogenesis in 3T3-L1 adipocytes at ambient temperatures of 37°C or 29°C as a marker for potential browning effects. The range of test concentrations of the two compounds was chosen based on the EC_{50} values for activation of TRPV1 (1.4 µM, NV [21]) or TRPA1 (63 µM, CA [22]), respectively. Negative effects of NV or CA in the applied concentrations on cellular proliferation as a measure for cell viability were excluded using standard MTT assays (data not shown). Neither incubation with 0.1-10 µM NV nor with 1-100 µM CA for 48 h at 37°C led to increased mitochondrial biogenesis (data not shown). However, when incubated at 29°C for 48 h, 0.1 µM NV as well as 10 µM CA increased the mitochondrial biogenesis by 99.2 ± 16.2% or 58.5 ± 16.2%, respectively. A very recent report on increased markers of thermogenesis at 37°C after treatment with CA was carried out with higher test concentrations of 400 µM CA. Induction of thermogenesis markers was analysed after short-term incubation up to 4 h [11], although longer-lasting effects have not been investigated yet on the mitochondrial level. Thus, it cannot be excluded that higher test concentrations of CA will lead to an increased number of brite adipocytes at 37°C as studied here. Data on the effect of NV on markers of thermogenesis in adipocytes are not available so far, however, the more pungent structural analogue of NV, capsaicin treatment has been shown to increase expression of genes associated with a brite adipocyte phenotype by Baboota *et al.*[23],

however, without addressing mitochondrial biogenesis directly. Overall the current data emphasize the need for more studies to evaluate the efficacy of food-derived TRP agonists like NV and CA to stimulate mitochondrial biogenesis at support of body weight loss.

To summarize, the presented data hint towards a long-term beneficial effect of the TRP channel agonists NV and CA on thermogenesis in addition to a cold-stimulus. Future studies are needed to confirm browning effects *in vitro* and *in vivo* and to clarify an involvement of TRPV1 and TRPA1.

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