Dynamic of lipid droplets and gene expression in response to β-aminoisobutyric acid treatment on 3T3-L1 cells

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Abstract

Research on adipobiology has recognized the browning process of white adipocytes as a potential therapeutic strategy for the treatment of obesity and related morbidities. Physical exercise stimulates the secretion of myokines, such as β-aminoisobutyric acid (BAIBA), which in turn promotes adaptive thermogenesis. Recent studies showed that the treatment with browning promoting factors in WAT. The exercise-induced adaptive thermogenesis would not make sense in white adipose tissue (WAT), since exercise per se increases heat production. Therefore, myokines, such as irisin, myostatin, meteorin-like 1 (Mettl1) and β-aminoisobutyric acid (BAIBA), which are released during muscle contraction in physical activity, are claimed as browning-promoting factors in WAT.

BAIBA is a non-protein amino acid originating from the catabolism of thymine and valine that plays a critical role in browning and in insulin resistance. Recently, BAIBA has been shown to promote osteocytes against reactive oxygen species and related apoptosis. Despite the role of myokines in WAT browning has been extensively reviewed, the cellular pathways through which they exert their action are mostly unknown. Roberts and colleagues hypothesized a PPARα-mediated mechanism through which BAIBA positively affects lipid metabolism in both adipocytes and hepatocytes. However, the exact molecular pathway of browning, as well as the remodelling of LDs induced by BAIBA needs further investigation.

This paper aims to show the effects of BAIBA treatment on murine 3T3-L1 preadipocytes during their differentiation to mature adipocytes on morphological and numerical changes of LDs and on the expression of a selected gene pattern. The expression of a core set of brown fat-specific genes and mitochondrial biogenesis (Ucp1, Cidea, Elov3, Ppargc1a, Ppara-g1a, Ppardn16 and Cyc1) and a beige marker (Tbx1) was analyzed. Markers of LD formation and dynamics such as perilipin 1 (Plin1) and cell death-inducing DFFA-like effector C (Cidec) were investigated. Finally, the expression of brain-derived neurotrophic factor (Bdnf), a neurotrophin, which has a role in browning on in vivo models and in regulating energy balance and insulin signaling, was also assessed.
Materials and Methods

Chemicals and culture media

 Dulbecco’s modified Eagle medium (DMEM) enriched with 4.5 g/L D-glucose, 110 mg/L sodium pyruvate and 862 mg/L L-alanyl-L-glutamine (Glutamax™), DMEM/F-12 (1:1) medium enriched with Glutamax™ and 15 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), penicillin/streptomycin solution and amphotericin B solution were purchased from Gibco by Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI, USA). BAIBA was purchased in a racemic mixture from AdipoGen SA (AdipoGen Life Science Inc., San Diego, CA, USA). Dipyromethene boron difluoride (BODIPY) 493/503 dye, TRIZol reagent, PureLink™ RNA Mini Kit and Platinum™ SYBR™ Green qPCR SuperMix-UDG kit for real time PCR were purchased from Invitrogen (Thermo Fisher Scientific Inc.). ImPront-II™ Reverse Transcription System was purchased from Promega (Madison, WI, USA). 4,6-diamidino-2-phenylinodole (DAPI)-containing mounting medium was purchased from Abcam (Cambridge, MA, USA). All other chemicals used in the experiment and not listed above were purchased from Sigma-Aldrich (Darmstadt, Germany).

Cell culture and treatment

3T3-L1 cells were chosen in the present study for their extensive use in evaluating the effects of compounds or nutrients on adipogenesis and in the potential application of various compounds and nutrients in the treatment of obesity. Moreover, at the best of our knowledge, the browning effect of BAIBA on 3T3-L1 cell model was never investigated. The murine 3T3-L1 preadipocytes (ZenBio Inc., Durham, NC, USA) were grown in high glucose DMEM supplemented with 10% FBS, 1% amphotericin B solution and 1% penicillin/streptomycin solution, at 37°C with 5% CO₂ and 95% relative humidity. Differentiation was induced 48 h after cells reached full confluence with DMEM/F-12 medium supplemented with 10% FBS, 1% amphotericin B solution, 1% penicillin/streptomycin solution, 0.5 μg/mL human insulin, 5 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 1 mM triiodothyronine (T₃) and 1 μM rosiglitazone. After 3 days, the differentiation medium was switched to a maintenance medium to which 0.5 μg/mL human insulin and 1 nM T₃ were added. Maintenance medium was refreshed every 2 days. After the switch from differentiation to maintenance medium, cells were treated with 1, 3 and 5 μM BAIBA until analysis. Cells were analyzed during differentiation at day 4 (4d) and at day 8 (8d), at day 10 (10d); these time points correspond to 2, 6 and 8 days of BAIBA treatment, respectively. A negative vehicle control (CTRL) was established treating cells with sterile milliQ water.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Medium was removed from cells plated on a 96-well plate and treated with different concentrations of BAIBA. Prior to incubation with 5 mg/mL MTT solution in Hank’s balanced salt solution (HBSS), cells were rinsed with phosphate buffer saline (PBS) 1x. Incubation with MTT solution was performed at 37°C for 4 h. Accumulated formazan was dissolved in dimethyl sulfoxide (DMSO) and incubated overnight at 37°C. The optical density was used as an indicator of cell viability and was measured at 550 nm.

BODIPY staining

After cell fixation in a 2% formalin solution diluted in PBS 1x at room temperature (RT) for 15 min, the slides were washed three times in PBS 1x and then incubated in a solution of BODIPY493/503 in PBS 1x to fluorescently label lipid droplets. The incubation was performed at RT in dark for 45 min. After the incubation, the slides were washed in PBS 1x three times and then mounted with mounting medium with DAPI. Images were acquired with the fluorescence microscope Axio Observer Z1 equipped with D-PLAN Neofluar objective lenses with N.A. 0.75 and Infinity Color Corrected System (ICS) and with AxioCam and Zen blue software (Carl Zeiss, Jena, Germany). The filters used for BODIPY were 470/525 nm, and 390/460 nm for DAPI.

Morphology of LDs

MRI Lipid Droplets tool (http://dev.mri.cars.fsu.edu/projects/imagej-macros/wiki/Lipid Droplets Tool), a macro of ImageJ1.50b software (http://rsb.info.nih.gov/iij/), was used to measure LD area. The images were analyzed as already described. For each LD area, perimeter, maximum Feret diameter (MFD) and integrated optical density (IOD) were measured. MFD is defined as the maximum distance between the two parallel planes restricting the object perpendicular to that direction and is a measure of an object size along a specified direction. It is generally used in optical microscopy to measure the size of particles. The IOD reports the average intensity/density of each object and increases linearly with the triglycerides content in 3T3-L1 adipocytes. Total area occupied by LDs in each field at different days of differentiation was calculated by summing up the area of each LD. The count of cells in each field was used to calculate the area and the number of LDs per cell. The average area of a single LD was calculated by dividing the area of LDs/cells by the number of LDs/cells. The area (μm²) and number of LDs were measured and counted on 15 fields for each time point.

RNA extraction and RT-PCR

After culture medium removal from 6-well plates, 1 mL/10 cm² of TRIZol reagent was added in each well and repeatedly pipetted to induce a severe breakdown of cell structures. These samples were immediately processed with the PureLink™ RNA Mini Kit following the manufacturer’s instructions. The concentration of the extracted total RNA was quantified using a spectrophotometer (NanoDrop 1000 Spectrophotometer; ThermoScientific, Wilmington, DE, USA) and the purity of RNA samples ranged between 1.8 and 1.9. RNA integrity was evaluated through the observation of 18S and 28S ribosomal bands after electrophoresis on 1% agarose gel, in the presence of GelRed. Primer3 Input software was used to design primers. GenBank accession, primer sequences, product lengths and relative annealing temperatures for each gene are listed in Table 1, according to the HUGO Gene Nomenclature Committee. Total RNA (500 ng) from each sample was reverse-transcribed and amplified using an MJ thermal cycler (PT-100; MJ Research, Inc., Waltham, MA, USA). For each gene, an aliquot of CDNA samples were pooled and standard curves with serial dilution of pool were used to optimize PCR conditions and to calculate the efficiency.
fluorescence baseline and threshold. Real time PCRs were performed for each sample in triplicate form using Platinum® SYBR® Green qPCR SuperMix-UDG. cDNA concentrations and primers molarities were different for each gene and determined with standard curves analyses. PCR amplification was conducted applying 45 cycles (1 s at 95°C, 30 s at the specific annealing temperature, 30 s at 72°C) in a 96-well spectrophotometric thermal cycler (CFX, Biorad, Milan Italy). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression of target genes was normalized using the TATA box-binding protein (Tbp) mRNA levels or geometric means between Tbp and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) genes and analyzed using ΔΔCt method.23 For all the cell culture experiments, the results are generated from biological triplicates and represent similar results from at least three independent experiments.

### Statistical analysis

Results are presented as relative values (means ± SEM). All experiments were performed at least three times. Two-way ANOVA was used for statistical analysis for MTT test by SPSS ver. 20.0 software. The measurements of the area of LDs/cell, number of LDs/cell and the area of a single LD were analyzed by SPSS ver. 20.0 software. The results, obtained from ten biological replicates, were compared using Kruskal-Wallis statistical test followed by pairwise comparisons using the Mann-Whitney approach.

### Results

#### Cell viability

The percentage of cell viability after exposure of BAIBA at 4d, 8d and 10d was always around 100% (data not shown). No significant differences were observed for time point and for concentration x time interaction.

#### Lipid droplet distribution and analysis

Figure 1 illustrated LDs formation in 3T3-L1 cells at 4d, 8d and 10d in presence of different BAIBA doses. Interestingly, at 4d with 3 and 5 μM of BAIBA, cells showed very small LDs (Figure 1, panels g and i, respectively) in comparison to CTRL cells (Figure 1a).

Indeed, kernel density distribution suggested an high number of cells with a small Feret diameter when incubated with 5 μM of BAIBA after 4 and 10 days of differentiation (Figure 2a). The kernel distributions of MFD at 4d, 8d and 10d are shown in Figure 2a. For all the three time points, LD size distribution of the treated cells is similar to that of control cells. In particular, using the least squares method evaluated on 100 equally spaced Feret dimensions, the differences at 4d between control and the treatments (1 μM, 3 μM and 5 μM) are respectively 0.0045, 0.0047 and 0.0538; at 8d are 0.0106, 0.0075 and 0.0062; at 10d are 0.0367, 0.0807 and 0.0736. Figure 2b evidenced statistically significant differences of IOD, number of LDs/cell and area of MFD at 4d, 8d and 10d.

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**Table 1. Oligonucleotide primer sequences for RT-PCR.**

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<thead>
<tr>
<th>Gene</th>
<th>GenBank accession</th>
<th>Primer sets</th>
<th>Product length (bp)</th>
<th>Tm (°C)</th>
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<tr>
<td>Tbp</td>
<td>NM_013684.3</td>
<td>Forward: 5'‐CCATGACCTTCTGTACCCCTA‐3'</td>
<td>104</td>
<td>58.5</td>
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<tr>
<td>Gapdh</td>
<td>NM_008984</td>
<td>Reverse: 5'‐CAGCCAGTACCCCTGTAGA‐3'</td>
<td>172</td>
<td>64.9</td>
</tr>
<tr>
<td>Ucp1</td>
<td>NM_008963.3</td>
<td>Forward: 5'‐CTTGCCTACTAAAGGCTTG‐3'</td>
<td>123</td>
<td>59.8</td>
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<tr>
<td>Pparα</td>
<td>NM_008942.2</td>
<td>Reverse: 5'‐TTGGCAAAAGGAGGTGTC‐3'</td>
<td>191</td>
<td>60.0</td>
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<tr>
<td>Cidec</td>
<td>NM_007702.2</td>
<td>Forward: 5'‐ATCACAATTCCGCTTACCC‐3'</td>
<td>136</td>
<td>58.9</td>
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<tr>
<td>Cidea</td>
<td>NM_178373.4</td>
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<td>Elovl3</td>
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<td>Reverse: 5'‐GGCCAACAACGATGACAGC‐3'</td>
<td>139</td>
<td>58.9</td>
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<td>Cyc1</td>
<td>NM_025567.3</td>
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<td>Plin1</td>
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<td>Reverse: 5'‐TTGCAACCTCGAGAGAG‐3'</td>
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<td>Prdm16</td>
<td>NM_027504.3</td>
<td>Reverse: 5'‐GCCACCGCCAAGCTTCC‐3'</td>
<td>110</td>
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<td>Ppara</td>
<td>NM_011144.6</td>
<td>Forward: 5'‐CTCAGACCACTGAGTGCA‐3'</td>
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<td>Bdnf</td>
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<td>Tbx1</td>
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<td>Reverse: 5'‐GAGGAGAAGAAGATGTTA‐3'</td>
<td>118</td>
<td>58.4</td>
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</table>

Tm, annealing temperature; Tbp, TATA box binding protein; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Ucp1, uncoupling protein 1; Pparα, peroxisome proliferator-activated receptor coactivator 1 α; Cidea, cell death inducing DFFA like effector A; Cidec, cell death inducing DFFA like effector C; Elovl3, elongation of very long chain fatty acids protein 3; Cyc1, cytochrome C1; Plin1, perilipin 1; Prdm16, proline rich domain containing 16; Ppara, peroxisome proliferator-activated receptor α; Bdnf, brain derived neurotrophic factor; Tbx1, T-box 1.
of a single LD/cell at 4d in presence of different BAIBA doses. To avoid any technical or biological variance due to cell number among slides, the total LD area was normalized to the number of cells.

The IOD significantly (P<0.05) decreased between 3 μM and 5 μM, the number of LDs/cell increased between CTRL and 1 μM (P<0.05) and between CTRL and 5 μM (P<0.01). The area of a single LD/cell was significantly different between CTRL and 5 μM (P<0.001). Figure 2b evidenced statistically significant differences in the area of LDs at 10d. In particular, the area was higher (P<0.05) in CTRL and 3 μM in comparison to 5 μM. No statistical differences were found at 8d.

The decrease in IOD, which is inversely related with the amount of triglycerides in LDs, the lowest area of a single LD and the increase of the number of LDs/cell observed with 5 μM BAIBA at 4d can account for a LD generation through the lipolytic release of fatty acids.

Transcription data analysis

Figure 3 indicated the relationship between the different concentrations of BAIBA and the significant differentially expressed genes at 4d and 8d, respectively. At 10d, gene expression did not display any significant variation. Only significant differentially expressed genes were showed. At 4d

![Image of lipid droplet pattern](image_url)

Figure 1. Lipid droplet pattern (stained with BODIPY 493/503) in fixed 3T3-L1 cells at 4d (a,d,g,l), 8d (b,e,h,m), and 10d (c,f,i,n). Control cells (a,b,c) and cells treated with 1 μM (d,e,f) and 5 μM BAIBA at 4d and BAIBA at 8d (l,m,n). Nuclear staining DAPI. Images are representative of n. 5 biological replicates. Scale bars: 20 μm.
Figure 2 a) Kernel distribution of the Feret of the lipid droplets at 4, 8 and 10 days of differentiation in control and BAIBA-treated cells. b) Lipid droplet measurements on 3T3-L1 in presence of different BAIBA doses: Integrated optical density (IOD), number of LDs per cell, area of single LD after 4 days of differentiation and total area of LD and after 10 days of differentiation. Box plots show median (horizontal lines), first-to-third quartile (box), and the extreme values with the interquartile range (vertical lines). The area measures are expressed in $\mu m^2$ and are the average of 10 fields of each concentration. ***P<0.001, **P<0.05.

Figure 3. Differentially expressed genes between BAIBA doses after 4 and 8 days of differentiation. a) Genes involved in browning (three biological replicates) at 4d. Tbx1, T-box 1; Ppargc1a, peroxisome proliferator-activated receptor $\gamma$ coactivator 1 $\alpha$; Ppara, peroxisome proliferator-activated receptor $\alpha$; Prdm16, proline rich domain containing 16; Bdnf, brain derived neurotrophic factor. b) Lipid droplets related genes at 4d. Cidea, cell death-inducing DFFA-like effector A; Plin1, perilipin 1. c) Genes involved in browning (three biological replicates) at 8d. Ucp1, uncoupling protein 1. Bars represent the mean plus/minus standard error of the mean (SEM). *P<0.05; **P<0.01; ***P<0.001.
the expression of Tbx1 was significantly (P<0.05) different between 5 μM and lower doses. No significant differences were found with CTRL, maybe due to the high variability at the 5 μM dose. Ppara, Prdm16, and Bdnf were up-regulated (P<0.002) at the same dose, while Ppargc1a displayed significant differences for 1 μM and 5 μM in comparison to CTRL and 3 μM (Figure 3a). Cidea was significantly upregulated (P<0.05) at 5 μM, whereas Plin1 showed the significantly (P<0.05) lowest expression at 5 μM in comparison to CTRL (Figure 3b). The expression of Ppara1a, Ucp1 and Ppara (P<0.001) was at its highest with 1 and 3 μM BAIBA doses in comparison to CTRL and 5 μM (Figure 3c).

Discussion

The present study reports the browning effect of BAIBA at different concentrations on 3T3-L1 cells at 4, 8 and 10 days of differentiation. It provides evidence showing the dynamics of LD morphology together with the expression of a panel of genes related to adaptive thermogenesis and LD formation. Although it is well established that this molecule, secreted by myocytes during exercise and circulating in blood, triggers the browning of WAT, no studies were conducted after its description by Roberts et al. in 2014. This study tested for the first time on 3T3-L1 cells known as reliable cellular model used in obesity researches, the browning effect of BAIBA by measuring variations in morphology of LDs that have recently attracted great interest as dynamic structures at the hub of lipid remodeling and plasticity. However, it was also established that this molecule, secreted by myocytes during exercise and circulating in blood, triggers the browning of WAT, no studies were conducted after its description by Roberts et al. in 2014. This study tested for the first time on 3T3-L1 cells known as reliable cellular model used in obesity researches, the browning effect of BAIBA by measuring variations in morphology of LDs that have recently attracted great interest as dynamic structures at the hub of lipid remodeling and plasticity. Different strategies have been employed to elucidate the molecular basis of LD remodeling, with Cidea, Bdnf, and Ppara demonstrating the most promising results. In the present study, the expression of BAIBA at 4d on 3T3-L1 cells increased 5 times higher than in control (Figure 3a). In fact, Bdnf expression increases in human skeletal muscle after exercise, suggesting a role as a myokine, on skeletal muscle metabolism, by enhancing local and peripheral fatty acid oxidation. The action of BAIBA at 4d on 3T3-L1 cells increased, and BAT in rodents and cows,38,39 where it could be involved in fat metabolism and in the activation of the sympathetic response. Moreover, mRNA and protein BDNF expression increases in human skeletal muscle after exercise, suggesting a role as a myokine, on skeletal muscle metabolism, by enhancing local and peripheral fatty acid oxidation. The action of BAIBA at 4d on 3T3-L1 cells increased, and BAT in rodents and cows,38,39 where it could be involved in fat metabolism and in the activation of the sympathetic response. Moreover, mRNA and protein BDNF expression increases in human skeletal muscle after exercise, suggesting a role as a myokine, on skeletal muscle metabolism, by enhancing local and peripheral fatty acid oxidation. The action of BAIBA at 4d on 3T3-L1 cells increased, and BAT in rodents and cows,38,39 where it could be involved in fat metabolism and in the activation of the sympathetic response. Furthermore, in vivo experiments showed that exercise training has marked effects on mitochondrial gene expression and activity in subcutaneous WAT in mice and human. Ppara controls mitochondrial biogenesis and heat production through induction of expression of Ucp1 and other respiratory factors. The higher expression level of this gene and Prdm16, a classic marker of brown fat, at 4d and later at 8d is coherent with the higher transcription of Ucp1 (Figure 3c).

Ppara, a nuclear receptor, stimulates genes involved in mitochondrial fatty acid oxidation and increases the expression of Ucp1 by directly interacting with Ppara1a in primary murine brown adipocytes. Roberts et al. demonstrated that browning process induced by BAIBA involved a specific Ppara-dependent mechanism, without clarifying the upstream mechanism of action. Very recently, it has been shown that the loss of Ppara affected mature brown adipocytes by reducing the expression of brown markers in vitro but not in vivo, suggesting as well an important role of glycogen kinase in maintaining the activity of PPAR proteins. Moreover, it should be considered that PPARα regulates Cidea in mouse liver without altering the expression of Ucp1. In the present paper, the expression of Ppara is induced by 5 μM BAIBA at 4d and its expression was further increased at 8d with the lowest BAIBA concentration, as well as the expression of Ppara1a and Ucp1 (Figure 3c). However, at 8d no significant variations in LD morphology and number were appreciated, as observed in PPARα2-programmed cells treated with BAIBA. At 10d, BAIBA treatments did not significantly affect gene expression, although a decrease in the area of LDs was detected at the highest dose (Figure 3d).
References


5. Riquier D. UCP1, the mitochondrial uncoupling protein of brown adipocyte: a personal contribution and a historical perspective. Biochimie 2017;134:3-8.


