Stimulation of uncoupling protein 1 expression by β-alanine in brown adipocytes

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Short title: Role of β-alanine in brown adipogenesis

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Abstract

Carnosine, which is abundant in meat, is a dipeptide composed of β-alanine and histidine, known to afford various health benefits. It has been suggested that carnosine can elicit an anti-obesity effect via induction and activation of brown/beige adipocytes responsible for non-shivering thermogenesis. However, the relationship between carnosine and brown/beige adipocytes has not been comprehensively elucidated. We hypothesized that β-alanine directly modulates brown/beige adipogenesis and performed an *in vitro* assessment to test this hypothesis. HB2 brown preadipocytes were differentiated using insulin from day 0. Cells were treated with various concentrations of β-alanine (12.5-100 μM) during adipogenesis (days 0-8) and differentiation (days 8-10). Then, cells were further stimulated with or without forskolin, an activator of the cAMP-dependent protein kinase pathway, on day 8 or day 10 for 4 h before harvesting. We observed that HB2 cells expressed molecules related to the transport and signal transduction of β-alanine. Treatment with β-alanine during brown adipogenesis dose-dependently enhanced forskolin-induced *Ucp1* expression; this was not observed in differentiated brown adipocytes. Consistent with these findings, treatment with β-alanine during days 0-8 increased phosphorylation levels of CREB in forskolin-treated HB2 cells. In addition, β-alanine treatment during brown adipogenesis increased the expression of *Pparα*, known to induce brown/beige adipogenesis, in a dose-dependent manner. These findings revealed that β-alanine could target HB2 adipogenic cells and enhance forskolin-induced *Ucp1* expression during brown adipogenesis, possibly by accelerating phosphorylation and activation of CREB. Thus, β-alanine, a carnosine-constituting amino acid, might directly act on brown adipogenic cells to stimulate energy expenditure.

Key words: β-alanine, brown adipocytes, UCP1, carnosine, CREB

Introduction

It is well-established that brown/beige adipocytes expend chemical energy as heat (Cannon and Nedergaard, 2004; Kajimura and Saito, 2014). Uncoupling protein 1 (UCP1) is a principal molecule responsible for energy expenditure in brown and beige adipocytes by uncoupling electron transfer with ATP production (Cannon and Nedergaard, 2004; Kajimura and Saito, 2014). It has been suggested that brown adipocytes mainly constitute brown adipose tissues located in the interscapular region of rodents (Cannon and Nedergaard, 2004). In contrast, beige adipocytes are induced in response to various stimuli, including cold exposure in white adipose tissues (Ishibashi and Seale, 2010). Both adipocytes increase the expression of UCP1 in response to activation of the cAMP-dependent protein kinase (PKA) pathway (Cannon and Nedergaard, 2004; Cohen and Kajimura, 2021).

Carnosine is a dipeptide composed of β-alanine and histidine and is found to be enriched in meat. Carnosine confers a variety of positive health effects (Wu, 2020). Reportedly, carnosine ingestion can alleviate increased oxidative stress, as well as the aggravated serum lipid profile resulting from intake of a high-fat diet (Yang et al., 2014). Al-Sawalha et al. (2019) have revealed the ameliorative effects of carnosine on obesity, along with improved blood pressure and blood glucose levels in rats fed a high-carbohydrate and high-fat diet. Furthermore, oral administration of carnosine can induce antidepressant-like effects in rats (Tomonaga et al., 2008).

Schaalan et al. (2018) have revealed that intraperitoneal administration of carnosine induced UCP1-positive adipocytes in white adipose tissues of rats fed a high-fat diet, and increased plasma levels of irisin, a myokine stimulating beige adipogenesis (Boström et al., 2012). Based on these findings, the authors speculated that carnosine-induced beige adipogenesis is indirectly induced by increasing muscular irisin production. Considering

that carnosine is hydrolyzed to its amino acids by carnosinase in the circulation and liver of rats (Lenney, 1976; Nagai et al., 2003; Everaert et al., 2012), β-alanine or histidine could stimulate the induction of UCP1-positive adipocytes. Accordingly, we hypothesized that carnosine-constituting amino acids directly act on brown/beige adipocyte lineage cells. In the present study, we examined the role of β-alanine during brown adipogenesis and in brown adipocytes.

Materials and methods

Materials

The reagents were purchased as follows: β-alanine (cat. no: 014-01062) and forskolin (cat. no: 067-02191) were from FUJIFILM Wako Pure Chemical (Osaka, Japan); insulin (cat. no: 16634) was from Sigma (St. Louis, MO, USA); Sepasol-RNA I Super G (cat. no: 09379), ReverTra Ace qPCR RT Master Mix (cat. no: FSQ-201), THUNDERBIRD SYBR qPCR Mix (cat. no: QPS-201), Can Get Signal 1 (cat. no: 12663-44), and Can Get Signal 2 (cat. no: 12663-54) were from Toyobo (Osaka, Japan); Immobilon-P (cat. no: IPVH00010) was from Merck (Tokyo, Japan); EzBlock Chemi (cat. no: AE-1475) was from Atto (Tokyo, Japan); antibodies against AMPKα (#2603), β-actin (#4967), ERK (#9102), JNK (#9252), p38 (#9212), phospho-AMPKα (#2535), phospho-CREB (Ser133) (87G3) (#9198), phospho-ERK (Thr202/Tyr204) (#9101), phospho-JNK (Thr183/Tyr185) (#9251), phospho-p38 (Thr180/Tyr182) (28B10) (#9216), phospho-SMAD1/5/8 (Ser463/Ser465) (D5B10) (#13820), and SMAD1 (D59D7) (#6944) were from Cell Signaling Technology (Danvers, MA, USA); antibody against ATF1 (25C10G) (SC-270), which also detects CREB1, was from Santa Cruz Biotechnology (Dallas, TX, USA); Chemi-Lumi One Ultra (cat. no: 11644-40) was from Nacalai Tesque (Kyoto, Japan).

Cell culture

HB2 brown preadipocytes isolated from the interscapular brown fat of p53-null mice (Irie et al., 1999), kindly provided by Dr. M. Saito, were cultured and differentiated as previously described (Suzuki et al., 2019). Two days after achieving desired confluency (day 0), HB2 cells were differentiated into adipocytes using a differentiation medium, comprising Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, and insulin (20 nM). Then, β-alanine (0, 12.5, 25, 50, or 100 μM) was added to the differentiation medium on days 0-8 or 8-10. On day 8 or 10, cells were treated with or without forskolin (10 μM), an activator of the PKA pathway, for 4 h prior to harvesting.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolation from HB2 cells by using Sepasol-RNA I Super G. Total RNA that was quantified by absorbance at 260 nm was reverse transcribed by use of ReverTra Ace qPCR RT Master Mix. The cDNA corresponding to 7.5-15 ng of total RNA was used as a template of qPCR; the qPCR was performed using THUNDERBIRD SYBR qPCR Mix in Thermal Cycler Dice Real Time System TP700 (TaKaRa, Otsu, Japan), according to the manufacturer's protocol. The qPCR profile was as follows: after denaturing for 30 s at 95 °C, 40 cycles consisted of 5 s at 95 °C and 30 s at 60 °C. The nucleotide sequences of qPCR primers are listed in Table S1. After 40 cycles of RT-qPCR, the dissociation (melting) curve of the products was examined by changes in the ramp temperature from 60 °C to 95 °C. Each sample showed a single peak, suggesting the expected PCR products. Expression levels of molecules related to transport and signal transduction of β-alanine were evaluated by assessing differences in cycle threshold (Ct) values between the target gene and TATA-binding protein (*Tbp*), i.e., 2 to the power of minus ΔCt that is subtracted Ct value of *Tbp* from Ct value of the target gene. In addition, the relative gene expression in cells treated with β-alanine and forskolin was examined with the ΔΔCt method, which

was used to normalize the levels of target transcripts to *Tbp* levels (Duran et al., 2005).

Western blot analysis

Cells were lysed in the mixture (50 μL/well) of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 1% (v/v) aprotinin, 1 mM Na3VO4; 35 μL) and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (350 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, 1 mM dithiothreitol; 15 μL), followed by heating for 10 minutes at 98°C. Six microliters of the cell lysates were subjected to SDS-PAGE using a 10% polyacrylamide gel, followed by blotting to PVDF membranes (Immobilon-P). The membranes were blocked with EzBlock Chemi for 4 hours at room temperature. Primary antibody was diluted at 1:1000-5000 in antibody dilution reagent (Can Get Signal 1). The membranes were reacted with the primary antibody for 16 hours at room temperature, followed by wash of the membrane with TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20) three times. Subsequently, the membranes were reacted with horseradish peroxidase -linked secondary antibody diluted at 1:200000 in Can Get Signal 2 for 45 minutes at room temperature, followed by wash with TBST three times. The immunoreactive proteins were visualized using Chemi-Lumi One Ultra according to the manufacturer's protocol. The luminescence was captured by LAS-4000 mini (GE Healthcare, Tokyo, Japan), and the band intensity was quantified by use of Image J software (https://imagej.nih.gov/ij/). Relative band intensity in cells treated without β-alanine was set at 1.

Statistical analysis

Data are expressed as the mean ± SEM. Data on gene expression and band intensity of western blot were log-transformed to provide an approximation of normal distribution before the analysis. Statistical analyses of gene expression were performed using the

GraphPad Prism (version 6.07; GraphPad Software, San Diego, CA). Data were analyzed by one-way or two-way analysis of variance (ANOVA). When the effect of β-alanine in one-way ANOVA or the effect of the interaction between β-alanine and forskolin in two-way ANOVA was significant ($P < 0.05$), comparisons among groups were performed using Tukey's test.

Results and discussion

Using RT-qPCR analysis, we evaluated the expression of molecules related to transport and signal transduction of β-alanine in HB2 cells on day 0 (Fig. 1A). In addition, the effect of β-alanine treatment on days 0-8 was examined (Fig. 1B-F). The uptake of β-alanine is mediated via the taurine transporter (TauT) (Usui et al., 2013) and γ-aminobutyric acid (GABA) transporter (GAT2) (Lopez-Corcuera et al., 1992). Reportedly, β-alanine activates GABA (Pan et al., 2005) and glycine receptors (Balse et al., 2006). In addition, β-alanine activates Mas-related G-protein coupled receptor D (MRGD) (Milasta et al., 2006). In the present study, *TauT*, GABA receptors (*Gabbr1* and *Gabrr1*), and glycine receptors (*Glrb* and *Glra1*) were expressed in undifferentiated HB2 cells; however, expression of *Gat2*, *Glra4* (a glycine receptor), and *Mrgd* failed to reach significant levels. Expression levels of *Glrb* and *Glra1* were decreased and increased after differentiation, respectively, but β-alanine treatment did not impact expression levels of the β-alanine transporter and receptor (Fig. 1B-F). These results suggest that β-alanine potentially modulates the differentiation or function of HB2 cells directly.

Moreover, we examined the effect of β-alanine treatment performed during days 0-8 on the expression of adipogenesis-related genes (Fig. 2A-C). As expected, differentiation stimulation markedly increased the expression levels of *Pparγ2* and *Fabp4*, markers of differentiated adipocytes (Fig. 2A, B). Expression levels of *Pparα*, which stimulates

brown/beige adipogenesis (Seale, 2015), were also increased after differentiation (Fig. 2C). Next, we evaluated the expression levels of genes related to brown/beige adipocytes (Fig. 2D-I). The expression levels of *Cox7a1* and *Tfam* were significantly increased after cell differentiation (Fig. 2E, F). Treatment with β-alanine during adipogenesis increased the expression of *Pparα* in a dose-dependent manner (Fig. 2C). Expression levels of the other genes were unaltered following treatment with β-alanine.

Reportedly, activation of the PKA pathway rapidly increases the expression of *Ucp1* in brown/beige adipocytes (Cannon and Nedergaard, 2004, Wu et al., 2012). Two-way ANOVA revealed significant interaction between β-alanine effect and forskolin effect. Thus, we evaluated effect of β-alanine in cells treated with and without forskolin separately. Treatment with β-alanine did not affect the basal expression of *Ucp1* but enhanced forskolin-induced expression levels of *Ucp1* (Fig. 3A); this indicates that the ratio of *Ucp1* expression levels in forskolin-treated cells to the basal *Ucp1* expression levels was dose-dependently increased following treatment with β-alanine (Fig. 3B). Thus, β-alanine potentiated to differentiate into brown adipocytes that are more responsive to PKA stimulation. Provided that Ucp1 is the gene responsible for thermogenesis, β-alanine may be helpful to enhances adaptive thermogenesis.

Activation of the PKA pathway stimulates phosphorylation and activation of cAMP-response element binding protein (CREB) (Johannessen et al., 2004), which is known to be responsible for transcriptional activation of the *Ucp1* gene by binding to its promoter (Cannon and Nedergaard, 2004). Consistent with results regarding *Ucp1* expression, forskolin-induced phosphorylation of CREB was dose-dependently enhanced following β-alanine treatment (Fig. 4A, Supplementary Fig. S1). According to the datasheet of antibodies for phosphorylated CREB (https://www.cellsignal.jp/datasheet.jsp?productId=9198&images=1), this antibody also

detects phosphorylated ATF1 at approximately 38 kDa. β-Alanine treatment did not significantly increase ATF1 phosphorylation levels in forskolin-treated cells. Treatment with β-alanine did not modulate the phosphorylation status of ERK, p38, JNK, SMAD1/5/8, and AMPKα in HB2 cells treated without forskolin (Fig. 4B, Supplementary Fig. S2).

We also examined the effect of β-alanine on differentiated HB2 cells; β-alanine treatment was performed from days 8-10. Expression levels of brown/beige adipocyte-related genes were unaltered following β-alanine treatment, except for *Cidea*, which was decreased by β-alanine treatment (Fig. 5). In addition, unlike treatment during brown adipogenesis, treatment with β-alanine did not affect forskolin-induced *Ucp1* expression in differentiated brown adipocytes (Fig. 6A, B).

A previous study has shown that intraperitoneal carnosine can induce the development of beige adipocytes in mice, speculated to be an indirect effect resulting from increased muscle production of irisin (Schaalan et al., 2018). Herein, we undoubtedly showed that β-alanine, a constituent of carnosine, at ~100 μM directly enhanced *Ucp1* expression during brown adipogenesis in response to activation of the PKA pathway. Treatment of fibroblasts and cardiomyocytes with β-alanine (5 mM) reportedly impairs mitochondrial function (Shetewy et al., 2016). In humans, plasma β-alanine concentrations transiently approached approximately 400 μM after ingestion of chicken broth (Harris et al., 2006). In addition, drinking carnosine (60 mg/kg body weight) increases plasma β-alanine levels to nearly 500 μM (Everaert et al., 2012). Thus, β-alanine produced by ingestion of carnosine-rich foods is unlikely to impair mitochondrial function but can positively impact the activation of brown adipogenic cells.

It should be noted that the significant effect of β-alanine was limited to treatment

performed on days 0-8, and gene expression levels were unaltered when β-alanine treatment was performed on days 8-10. Thus, it is possible that β-alanine targets HB2 brown preadipocytes but not differentiated adipocytes, and β-alanine is potentially involved in regulating brown adipogenesis, but not in brown adipocytes. Cell stage-dependent effects have been reported in HB2 cells (Kida et al., 2016; Suzuki et al., 2018); treatment with 17β-estradiol during adipogenesis, but not in differentiated adipocytes, enhanced the responsiveness to isoproterenol, a β-adrenergic receptor agonist, following *Ucp1* induction (Suzuki et al., 2018). In contrast, high-dose capsaicin increased expression levels of *Fabp4*, *Pparγ2*, and *Pgc1α*, which were limited to the treatment of differentiated brown adipocytes (Kida et al., 2016).

Previous studies have revealed that β-aminoisobutyric acid (BAIBA) and taurine, molecules structurally related to β-alanine, activate brown/beige adipocytes (Roberts et al., 2014; Guo et al., 2019). Treatment with BAIBA induced the expression of *Pparα* in cultured adipocytes, and BAIBA-induced *Ucp1* expression was blocked following co-treatment with GW6471, a selective PPARα antagonist (Xu et al., 2002), in primary adipocytes. Furthermore, upregulation of *Ucp1* expression following BAIBA intake through drinking water could not be detected in *Pparα*-null mice (Roberts et al., 2014). These results are partly consistent with the present results: β-alanine increased the expression of *Pparα*, but basal *Ucp1* levels were unaltered following β-alanine treatment. Intraperitoneal administration of taurine reduced body weight gain and enhanced expression of *Ucp1* in inguinal fat of mice fed a high-fat diet. Furthermore, taurine increased the expression of *Ucp1* in cultured adipocytes via *Pgc1α* induction (Guo et al., 2019). The stimulating effects of BAIBA and taurine are distinct from those of β-alanine; β-alanine did not increase basal *Ucp1* expression but enhanced forskolin-induced *Ucp1* expression. Accordingly, β-alanine-related molecules may play a role in regulating the activation of brown/beige adipocytes via a molecule-dependent pathway.

Park and Cho (2006) have shown that *N*-acetyl-*O*-methyldopamine (NAMDA), an inhibitor of tetrahydrobiopterin synthesis (Cho et al., 1999), enhances forskolin-induced CREB phosphorylation in human neuroblastoma cells. The authors speculated that NAMDA-induced ERK phosphorylation could mediate the phosphorylation and activation of CREB (Park and Cho, 2006). Considering that β-alanine did not affect ERK phosphorylation in HB2 cells, the molecular basis underlying the enhancement of forskolin-induced CREB phosphorylation following β-alanine treatment differs from that mediated by NAMDA.

β1,4-*N*-acetylglucosaminyltransferase III (GnT-III), an enzyme that catalyzes the addition of a bisecting *N*-acetylglucosamine to *N*-glycans (Stanley, 2002), also increased forskolin-induced CREB phosphorylation in mouse neuroblastoma and melanoma cells (Li et al., 2007). Glycosylation of adenylate cyclase catalyzed by GnT-III can increase adenylate cyclase activity, resulting in enhanced responsiveness to forskolin. Future studies are needed to evaluate the effect of β-alanine on the glycosylation status and activity of adenylate cyclase in brown/beige adipogenic cells.

The present study reveals that β-alanine, a constituent of carnosine that is abundant in meat, directly stimulates differentiation into brown adipocyte with high responsiveness to PKA stimulation. Considering that effective concentration of β-alanine was within the range reaching after carnosine-containing food, it may be a practical tool to control adaptive thermogenesis in brown adipocytes.

Declarations

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent to publish

Not applicable

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Figure legends

Figure 1. Expression of molecules related to transport and signaling of β-alanine in HB2 cells

The expression of molecules related to the transport and signaling of β-alanine was examined in HB2 cells on day 0 (n=4) (A) or in HB2 cells on day 0 or day 8, following treatment with the indicated reagent (B-F). (A) 2 to the power of minus delta Ct that is subtracted Ct value of *Tbp* from Ct value of the indicated gene is shown (n=4). ND: not detected. (B-F) Expression levels in HB2 cells on day 0 were set at 1 (n=3-4). Data are presented as mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA, followed by Tukey's test. a, b: $P < 0.05$.

Figure 2. Expression of molecules related to brown/beige adipocytes in HB2 cells treated with β-alanine for days 0-8

The expression of molecules related to brown/beige adipogenesis was examined in HB2 cells on day 0 or in HB2 cells on day 8, following treatment with the indicated concentration of β-alanine (β-Ala) for days 0-8. The expression levels in HB2 cells on day 0 were set to 1 (n=4). Data are presented as mean ± standard error of the mean (SEM). Relative expression levels in cells on day 8 were analyzed by one-way ANOVA, followed by Tukey's test. a, b: $P < 0.05$.

Figure 3. Forskolin-induced *Ucp1* expression in HB2 cells treated with β-alanine for days 0-8

Ucp1 expression was examined in HB2 cells on day 0 and in HB2 cells treated with the indicated concentration of β-alanine (β-Ala) for days 0-8, followed by treatment with or without forskolin (10 μM) for 4 h. (A) Expression levels in HB2 cells on day 0 were set at 1 (n=3-4). ANOVA table is inserted. (B) Fold change, i.e., ratio of *Ucp1* level in forskolin-treated cells to that in forskolin-untreated cells, is shown (n=3-4). Data are

presented as mean ± standard error of the mean (SEM). (A) Relative expression levels in cells on day 8 were analyzed by two-way ANOVA. Because the interaction between β-alanine effect and forskolin effect was significant, comparisons among groups treated with or without forskolin were examined by Tukey's test. (B) The fold changes were analyzed by one-way ANOVA, followed by Tukey's test. a, b: $P < 0.05$.

Figure 4. Expression of signaling molecules in HB2 cells treated with β-alanine for days 0-8

HB2 cells were treated with the indicated concentration of β-alanine (β-Ala) for days 0-8, followed by treatment with or without forskolin (10 μM) for 4 h. (A) Phosphorylation of CREB/ATF1 was examined by western blot analysis. A representative image is shown (*left* panel). After quantification of the band intensity, relative band intensity was calculated and shown (n=3, *right* panel). The band intensity was analyzed by two-way ANOVA. Because the interaction between β-alanine effect and forskolin effect was significant, comparisons among groups treated with or without forskolin were examined by Tukey's test. a-c: Means that do not have a common letter on the bar statistically different ($P < 0.05$). (B) Phosphorylation of ERK, p38, JNK, SMAD1/5/8, and AMPKα was examined. A representative image is shown (*left* panel). After quantification of the band intensity, relative band intensity was calculated and shown (*right* panel).

Figure 5. Expression of molecules related to brown/beige adipocytes in HB2 cells treated with β-alanine for days 8-10

The expression of molecules related to brown/beige adipogenesis was examined in HB2 cells on day 10, following treatment with the indicated concentration of β-alanine (β-Ala) for days 8-10. Expression levels in control HB2 cells (untreated with β-alanine) were set at 1 (n=4). Data are presented as mean ± standard error of the mean (SEM). Relative expression levels in cells on day 8 were analyzed by one-way ANOVA, followed by

Tukey's test. a, b: $P < 0.05$.

Figure 6. Expression of *Ucp1* and signaling molecules in forskolin-treated HB2 cells treated with β-alanine for days 8-10

HB2 cells were treated with the indicated concentration of β-alanine (β-Ala) for days 8-10, followed by treatment with or without forskolin (10 μM) treatment for the final 4 h. (A, B) Expression of *Ucp1* was examined. (A) Expression levels in control HB2 cells (untreated without β-alanine and forskolin) were set at 1 (n=4). ANOVA table is inserted. (B) Fold change, i.e., ratio of *Ucp1* level in forskolin-treated cells to that in forskolin-treated cells, is shown (n=4). Data are presented as mean ± standard error of the mean (SEM). (A) Relative expression levels in cells on day 8 were analyzed by two-way ANOVA. (B) The fold changes were analyzed by one-way ANOVA, followed by Tukey's test. a, b: $P < 0.05$.

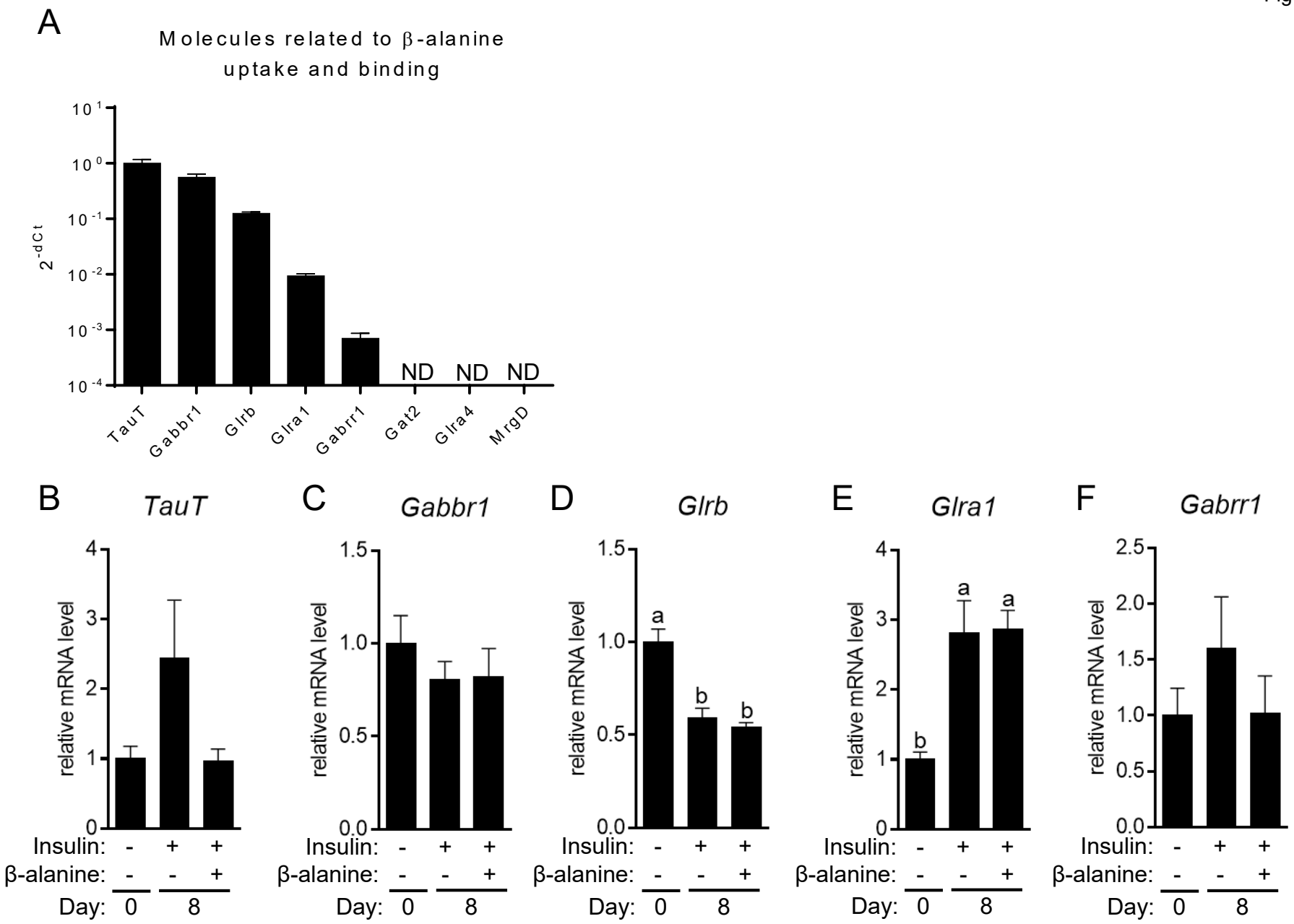
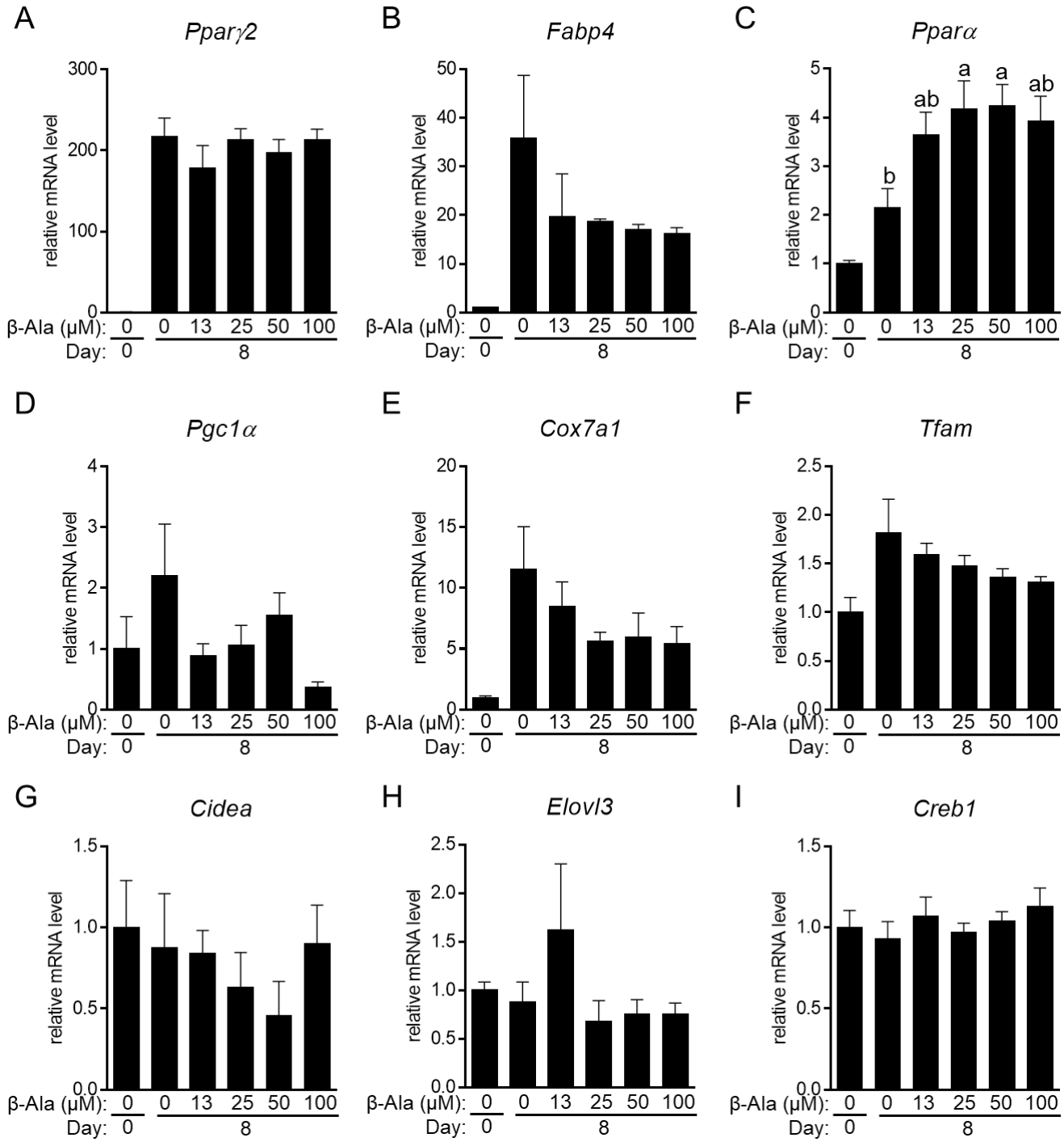
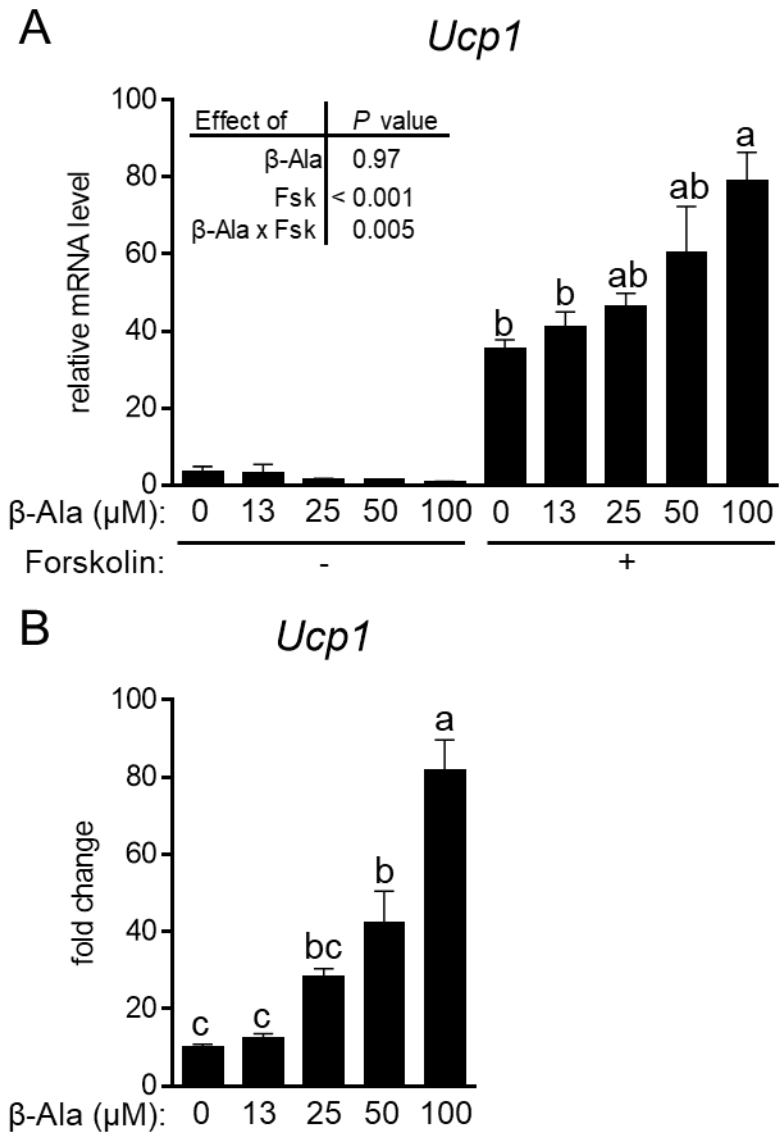
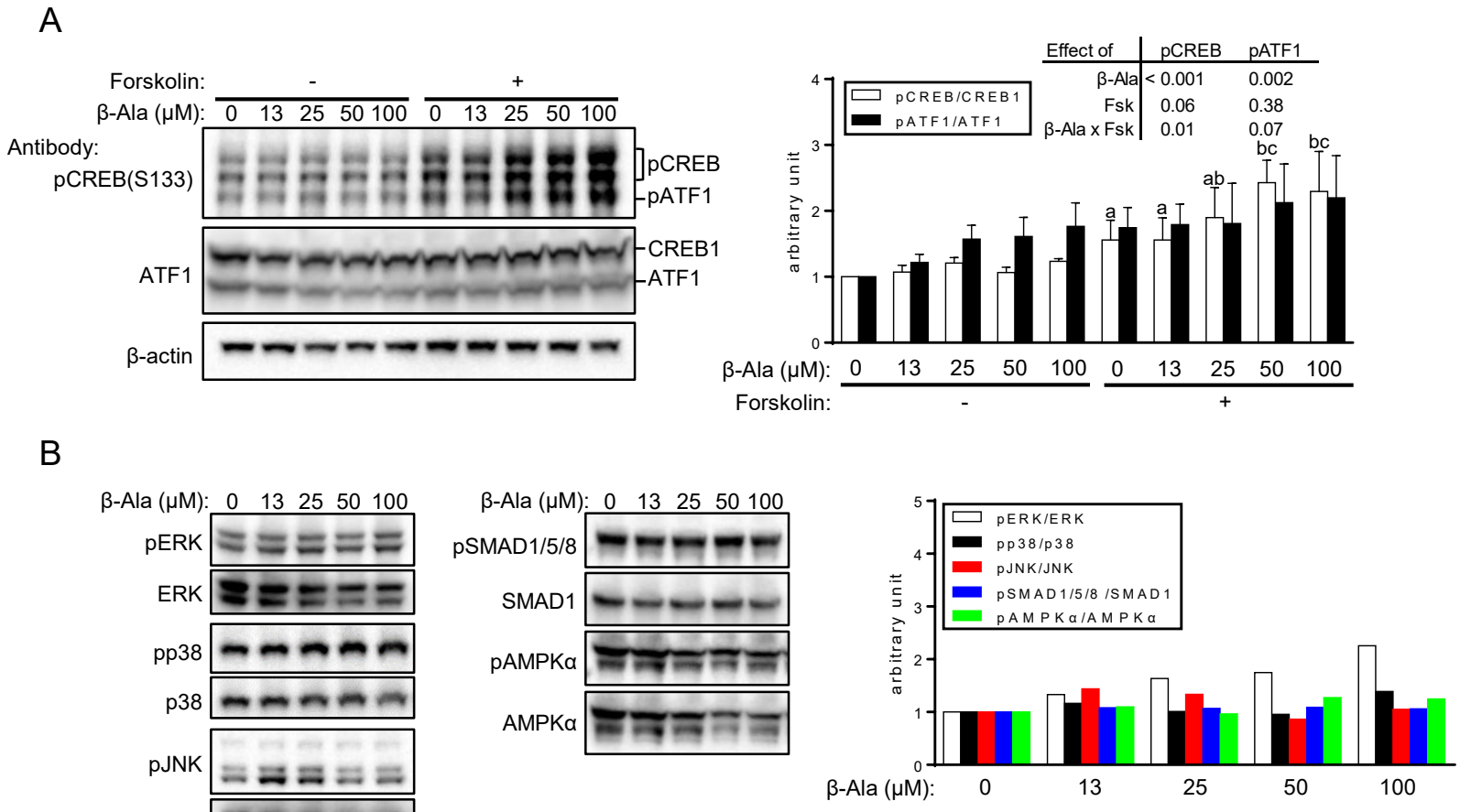

A
Molecules related to β-alanine uptake and binding
2^{-dCt}
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
ND ND ND
TauT
Gabbr1
Glrb
Glra1
Gabrr1
Gat2
Glra4
MrgD
B TauT
C Gabbr1
D Glrb
E Glra1
F Gabrr1
relative mRNA level
Insulin: - + +
β-alanine: - - +
Day: 0 8
a b b
b a a

Figure 2 (Hamaoka)




A
Ucp1
relative mRNA level
100
80
60
40
20
0
Effect of
P value
β-Ala
0.97
Fsk
< 0.001
β-Ala x Fsk
0.005
a
ab
b
ab
b
β-Ala (μM):
0
13
25
50
100
0
13
25
50
100
Forskolin:
-
+
B
Ucp1
fold change
100
80
60
40
20
0
a
b
bc
c
c
β-Ala (μM):
0
13
25
50
100


A
Forskolin: - +
β-Ala (μM): 0 13 25 50 100 0 13 25 50 100
Antibody:
pCREB(S133)
pCREB
pATF1
ATF1
CREB1
ATF1
β-actin
Effect of pCREB pATF1
β-Ala < 0.001 0.002
Fsk 0.06 0.38
β-Ala x Fsk 0.01 0.07
pCREB/CREB1
pATF1/ATF1
arbitrary unit
a a ab bc bc
β-Ala (μM): 0 13 25 50 100 0 13 25 50 100
Forskolin: - +
B
β-Ala (μM): 0 13 25 50 100
pERK
ERK
pp38
p38
pJNK
JNK
β-Ala (μM): 0 13 25 50 100
pSMAD1/5/8
SMAD1
pAMPKα
AMPKα
pERK/ERK
pp38/p38
pJNK/JNK
pSMAD1/5/8 /SMAD1
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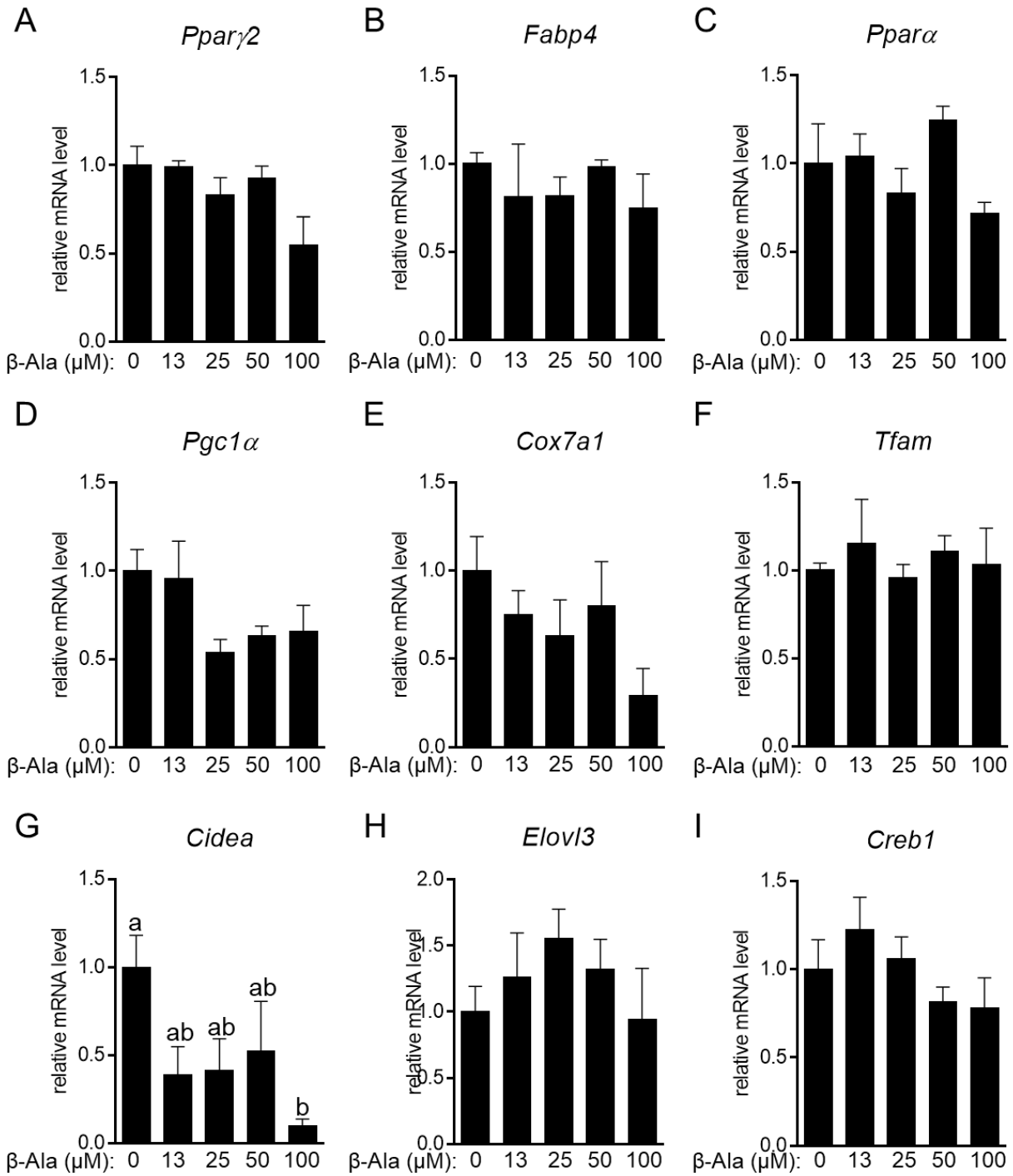
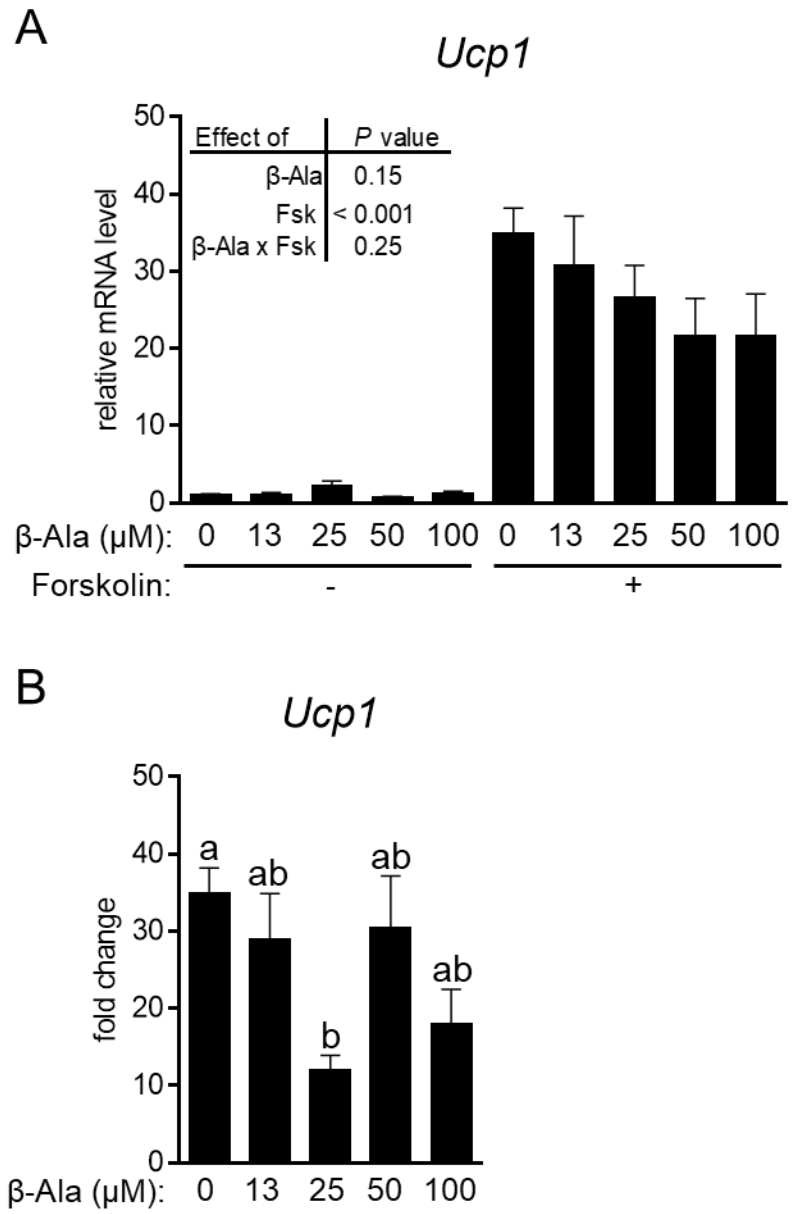
arbitrary unit
β-Ala (μM): 0 13 25 50 100


A
Pparγ2
B
Fabp4
C
Pparα
D
Pgc1α
E
Cox7a1
F
Tfam
G
Cidea
H
Elovl3
I
Creb1
relative mRNA level
β-Ala (μM): 0 13 25 50 100
a
ab
ab
ab
b


A
Ucp1
relative mRNA level
50
40
30
20
10
0
Effect of
P value
β-Ala
0.15
Fsk
< 0.001
β-Ala x Fsk
0.25
β-Ala (μM):
0 13 25 50 100 0 13 25 50 100
Forskolin:
-
+
B
Ucp1
fold change
50
40
30
20
10
0
a
ab
b
ab
ab
β-Ala (μM):
0 13 25 50 100

Supplementary information

Stimulation of uncoupling protein 1 expression by β-alanine in brown adipocytes

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Fig. 4A

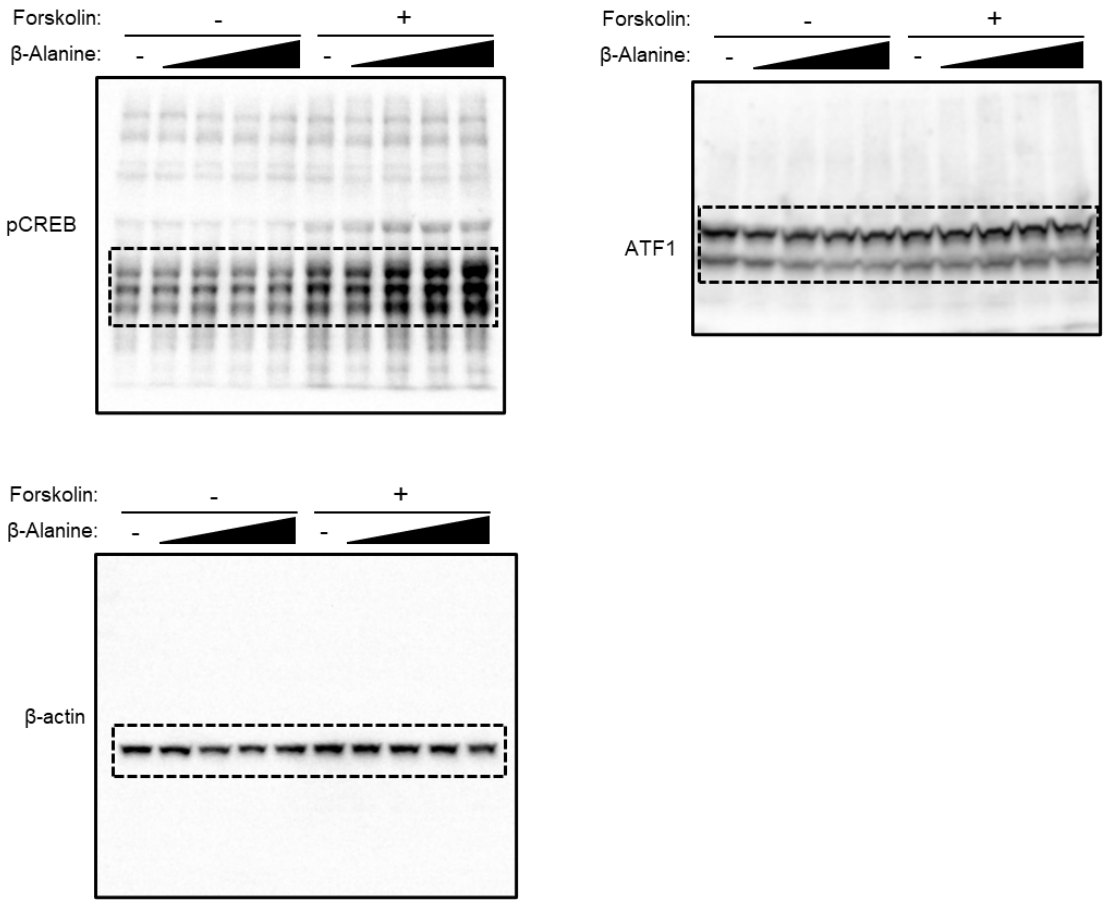

Figure S1. Images of full-length blot shown in Fig. 4A

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 4A.

Fig. 4B

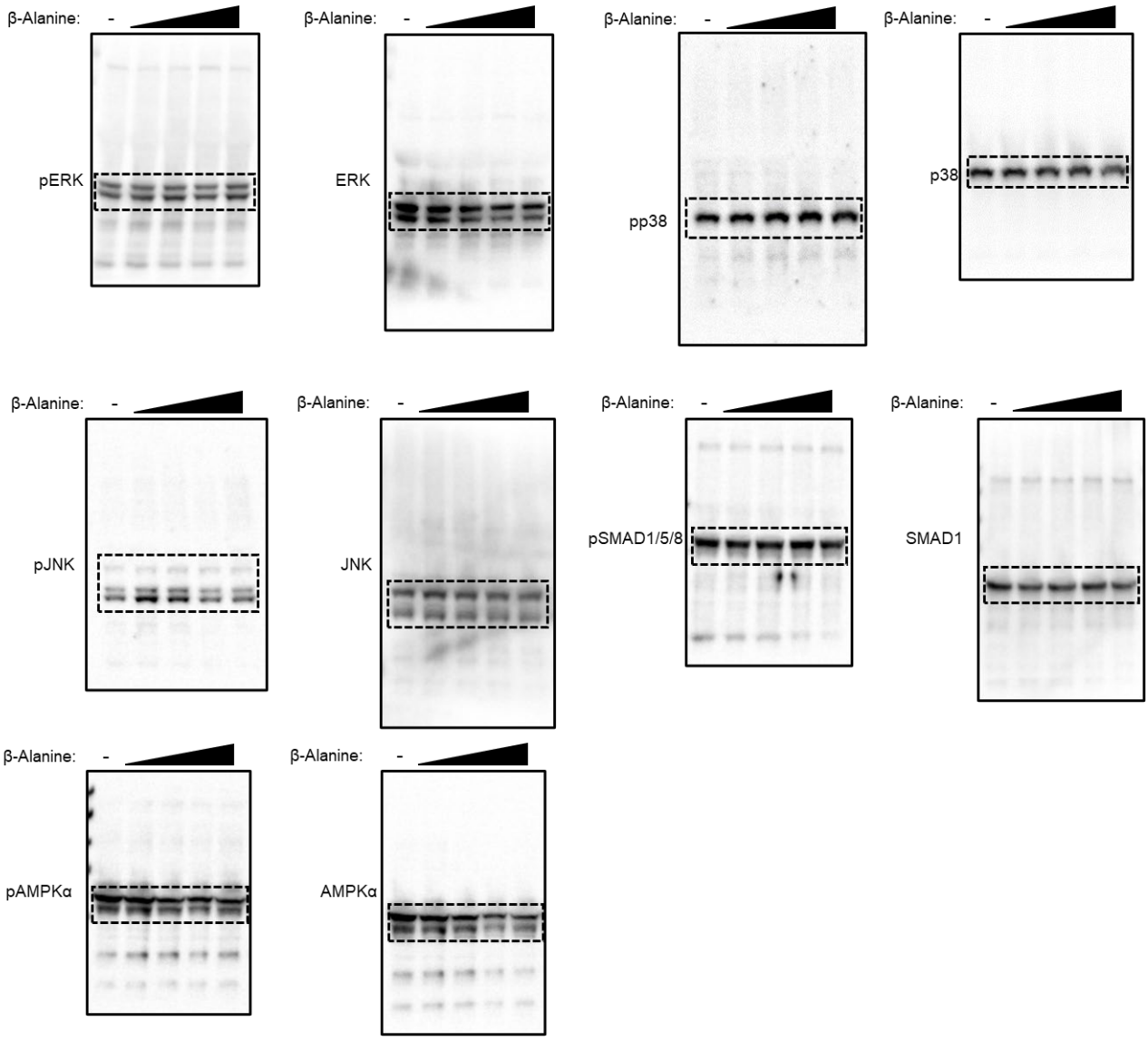

Figure S2. Images of full-length blot shown in Fig. 4B

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 4B.

Table S1. Oligonucleotide PCR primers for RT-qPCR

	5’-primer	3’-primer
Cidea	5’-AAACCATGACCGAAGTAGCC-3’	5’-AGGCCAGTTGTGATGACTAAGAC-3’
Cox7a1	5’-AAAGTGCTGCACGTCCTTG-3’	5’-TTCTCTGCCACACGGTTTTC-3’
Creb1	5’-GGAAGAGAGAGGTCCGTCTAATG-3’	5’-CACATATTCTTTCTTCTTTCTACGACA-3’
Elovl3	5’-ACTTCGAGACGTTTCAGGACTTA-3’	5’-GACGACCACTATGAGAAATGAGCTT-3’
Fabp4	5’-AAGGTGAAGAGCATCATAACCCT-3’	5’-TCACGCCTTTCATAACACATTCC-3’
Gabbr1	5’-CGAAGCATTTCCAACATGAC-3’	5’-CAAGGCCCAGATAGCATCATA-3’
Gabrr1	5’-ATGCTGTCCTGGGTGTCTTT-3’	5’-GTGATGATGGTGGACATGGT-3’
Gat2	5’-TGGAGGTTTCCCTATCTCTGC-3’	5’-GAAGAAGATGAAGTAGGGGATGAA-3’
Glra1	5’-GTTCCATCGCTGAGACAACC-3’	5’-GGGTATTCATTGTAGGCCAGAC-3’
Glra4	5’-TCCTCACCATGACAACTCAGA-3’	5’-TGTCAATTGCCTTTACGTAGGA-3’
Glrb	5’-CCAACTCCACCAGCAATATCT-3’	5’-TTGACTACTACATCAACAGGAATGC-3’
MtgD	5’-GCTGCTGGAAACACTTCTAGG-3’	5’-GGCTGCTGTCAAGAGTGGA-3’
Pgc1α	5’-TGTGGAACTCTCTGGAACTGC-3’	5’-GCCTTGAAAGGGTTATCTTGG-3’
Pparα	5’-AACTGGATGACAGTGACATTTCC-3’	5’-CCCTCCTGCAACTTCTCAAT-3’
Pparγ2	5’-TGCTGTTATGGGTGAAACTCTG-3’	5’-CTGTGTCAACCATGGTAATTTCTT-3’
TauT	5’-AAGAGCAAGGGGTGGACATT-3’	5’-AGCTTTTGGGTAGGCAATGA-3’
Tbp	5’-CCAATGACTCCTATGACCCCTA-3’	5’-CAGCCAAGATTCACGGTAGAT-3’
Tfam	5’-CAAAGGATGATTCGGCTCAG-3’	5’-AAGCTGAATATATGCCTGCTTTTC-3’
Ucp1	5’-CTTTGCCTCACTCAGGATTGG-3’	5’-ACTGCCACACCTCCAGTCATT-3’