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Visfatin expression analysis in association with recruitment and activation of human and rodent brown and brite adipocytes.

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Short title. Visfatin expression in brown adipocyte.

Abstract.

Human brown adipocytes are able to burn fat and glucose and are now considered as a potential strategy to treat obesity, type 2 diabetes and metabolic disorders. Besides their thermogenic function, brown adipocytes are able to secrete adipokines. One of these is visfatin, a nicotinamide phosphoribosyltransferase involved in NAD synthesis, known to participate in insulin synthesis by pancreatic β cells. In a therapeutic context, it is of interest to establish whether a potential correlation exists between brown adipocyte activation and/or brite adipocyte recruitment, and adipokine expression. We analyzed visfatin expression, as a pre-requisite to its secretion, in rodent and human biopsies and cell models of brown/brite adipocytes. We found that visfatin was preferentially expressed in mature adipocytes and that this expression is higher in brown adipose tissue of rodents compared to other fat depots. However, using various rodent models we were unable to find any correlation between visfatin expression and brown or brite adipocyte activation or recruitment. Interestingly, the situation is different in humans, where visfatin expression was found equivalent between white and brown or brite adipocyte *in vivo* and *in vitro*. In conclusion, visfatin can be considered only as a rodent brown adipocyte biomarker, independently to tissue activation.

Keywords.

Nampt, PBEF, hMADS, UCP1, obesity, type 2 diabetes.

Introduction.

Obesity participates for a great part in the pathogenesis of type 2 diabetes (T2D), due to its association with insulin resistance. Obesity has reached epidemic proportions worldwide, with more than 1.9 billion of adults suffering from overweight and 600 million considered as clinically obese ^{1, 2}. Unfortunately, except for morbidly obese patients undergoing bariatric surgery, medical obesity treatments are so far not satisfactory.

Overweight and obesity are consequent to energy balance disequilibrium, in favor of inappropriate energy intake increasing white fat mass. In this context, one of the strategies which deserves consideration is the use of the thermogenic capacity of brown adipocytes to counteract the excess of energy intake to equilibrate the energy balance ³. Brown adipocytes are characterized by a high mitochondrial content and expression of the specific uncoupling protein 1 (UCP1). These cells are endowed with a high capacity of glucose and lipid oxidation and thus making them a potential target for decreasing plasma glucose, fatty acids and lowering the risks of T2D and obesity ^{4, 5}. Indeed, the recent discovery of brown adipocytes, thermogenically competent, in human adults open a new field in obesity associated T2D therapy (reviewed in ⁶). In addition to brown adipose tissue (BAT) – consisting in brown adipocytes – white adipose tissue (WAT) contains thermogenic fat cells called “brown-in-white” (“brite”) or “beige” adipocytes ⁷. These brite adipocytes originate either from the differentiation of specific progenitors resident in the tissue ^{8, 9}, or from the conversion of differentiated white adipocytes ^{10, 11}. In addition to the preventive function of BAT in the occurrence of T2D, *i.e.* in fat gain and glucose tolerance regulation, this tissue seems to offer beneficial effects on metabolic disorders. Indeed, transplantation of BAT in mice resulted in a reversal of high-fat diet-induced insulin resistance ¹² and reverses streptozotocin-induced diabetes in mice ¹³. In healthy humans, cold exposure leads to a decrease in body fat by recruiting brown/brite adipocytes and increasing

nonshivering thermogenesis¹⁴⁻¹⁶. A recent study showed that these adipocytes may function as an anti-diabetic tissue in humans¹⁷. Altogether, these data explore new avenues emphasizing thermogenic adipocytes as novel important candidates to control body weight and carbohydrate metabolism through modulation of energy expenditure.

Visfatin (Nampt/PBEF) is an enzyme (nicotinamide-ribosyl-transferase) synthesizing nicotinamide mononucleotide (NMN) from nicotinamide¹⁸. NMN is the substrate for nicotinamide dinucleotide (NAD) synthesis, involved in numerous cell functions¹⁹. Visfatin, secreted from various cells including adipocytes, has been found in the circulation and is considered as an adipokine¹⁸. Several studies demonstrate the crucial role of visfatin in β -cell function, *i.e.* insulin secretion, insulin receptor phosphorylation and signaling as well as transcriptional regulation of various key genes^{18,20}. Thus, visfatin could be involved in metabolic disorders involving β -cell failure, such as T2D.

Since dysregulation of adipokine synthesis is associated with obesity, we hypothesize that brown adipocyte activation could participate in an increased production of visfatin and thus be involved in the regulation of insulin secretion and hence glucose metabolism. To evaluate visfatin as a potential target for future therapies of obesity and associated diseases, we analyzed here the expression of visfatin in several *in vivo* and *in vitro* models of brown adipocyte activation and brite adipocyte recruitment in rodents and humans.

Results.

Visfatin mRNA is preferentially expressed in adipocytes

Since adipose tissue is composed of adipocytes and several cell types of the stromal vascular fraction (SVF), we separated by enzymatic digestion these fractions from mouse adipose tissues isolated from interscapular (iBAT), subcutaneous (scWAT) and epididymal depots (eWAT). We measured mRNA expression of key adipocyte markers; *i.e.* perilipin-1 as control of adipocyte purification, and UCP1 as brown adipocyte marker. As expected, perilipin-1 and **leptin were** only expressed in the adipocyte fraction of the three adipose depots, while UCP1 mRNA expression was confined to the adipocyte fraction of iBAT (Fig. 1). In contrast to perilipin, **leptin** and UCP1, visfatin mRNA was expressed in both adipocyte and stromal vascular fractions, but with a two-fold higher expression in adipocyte fraction in the three adipose tissue depots (Fig. 1).

Visfatin is highly expressed in brown adipocytes

To determine whether visfatin expression was correlated to brown adipocyte differentiation, we analyzed its expression in *in vitro* differentiated mouse adipocytes. For that purpose, SVF cells from the three adipose tissue depots were induced to differentiate either into white **or brown/brite** adipocytes (Fig. 2A). As expected, UCP1 mRNA was not detected in white adipocytes, **leptin mRNA levels were low in brown/brite adipocytes**. In addition, UCP1 was highly expressed in brown adipocytes derived from iBAT progenitors, lower in brown adipocytes derived from scWAT progenitors (considered as brite adipocytes) and not detected in those derived from eWAT (Fig. 2A). Conversely, visfatin mRNA was expressed in SVF differentiated either in white or brown/brite adipocytes with a significantly higher expression in iBAT-derived brown adipocytes. **Then, we analyzed visfatin secretion in conditioned media and found that secreted visfatin protein levels were correlated to visfatin mRNA expression, with a higher secretion in**

brown adipocytes (Fig. 2B). Furthermore, visfatin secretion was not affected by β 3-adrenergic receptor stimulation (Fig. 2B).

Visfatin is preferentially found in iBAT, but its expression is not correlated with BAT activation

To determine whether a correlation exists between visfatin mRNA expression and BAT activation, mice were exposed to cold (4°C, 4 days) or received a daily injection of CL316,243 for 1 week, known to activate brown adipocytes. These stimulations induced a significant increase in UCP1 mRNA levels in iBAT. However, this was not correlated to an increase in visfatin mRNA expression (Fig. 3A and B). As expected, both cold and β 3-receptor agonist treatments led to an increased UCP1 expression in scWAT, but not in eWAT (Fig. 3A and B) corresponding to the recruitment and activation of inducible brite adipocytes in the scWAT. Again, visfatin mRNA levels were not significantly modified under these stimulations (Fig. 3A and B). Interestingly, a significant increase of visfatin mRNA was observed in eWAT under both stimulations (Fig. 3A and B).

Visfatin mRNA expression was higher in mice iBAT compared to white depots, *i.e.* eWAT and scWAT (Fig. 3A and B). A similar profile was found in rat tissues with a higher expression in iBAT compared to eWAT (Fig. 4A and B). Concerning the correlation with BAT activation in rat adipose tissue depots, no significant increase in visfatin mRNA levels was observed upon CL316,243 stimulation (Fig. 4B). Under acute cold exposure, visfatin expression decreased (Fig. 4A).

Visfatin is expressed and secreted by human brown and brite adipocytes.

We further analyzed the expression of visfatin in scWAT and BAT biopsies from healthy volunteers (evaluated by FDG-PET/CT analysis). UCP1 mRNA expression was confined to BAT, whereas visfatin mRNA was expressed in both tissues at similar levels (Fig. 5A).

We analyzed the expression of visfatin mRNA in two human cellular models. SVF from two human subcutaneous adipose tissue samples (hADSC) were induced to differentiate into white or brown adipocytes. As expected, UCP1 mRNA expression was restricted to brown adipocytes and **leptin mRNA was highly expressed in white adipocytes**. By contrast, visfatin mRNA was expressed at similar levels in both white and brown adipocytes (Fig. 5B). Next, we used hMADS cells, which are able to differentiate into white adipocytes and then convert into functional brite adipocytes. Whereas UCP1 mRNA expression was confined to brite adipocytes, visfatin mRNA was expressed in both cell types, brite adipocytes showing an expression two times higher than white adipocytes (Fig. 5C). To determine a potential functional impact of this up-regulation, we analyzed the secretion of visfatin in the conditioned media. We found that secreted visfatin protein levels, evaluated to be 15% of the total visfatin (cellular + secreted), were equivalent between white and brite hMADS adipocytes, even in the presence of CL316,243 (Fig. 5D). The increased visfatin mRNA expression might augment only the intracellular visfatin level as a slight, but not significant, increase was found in cell lysates of brite adipocytes (0.053 ± 0.016 ^{vs} / 0.088 ± 0.034 ng visfatin/ μ g protein respectively for white and brite adipocytes).

Discussion.

Visfatin is an essential NMN/NAD biosynthetic enzyme considered as a novel adipokine, with both intra- and extracellular enzymatic functions. It is expressed in white and brown adipose tissues of rodents ¹⁸. Secreted visfatin acutely potentiates glucose stimulated insulin secretion, without affecting β -cell survival ²¹. Moreover, NMN, the product of visfatin activity, has been shown to restore insulin secretion in mouse models of impaired β -cell function ²². Finally, visfatin is found in the blood circulation acting like a cytokine. Thus, targeting visfatin function or expression might represent a novel therapeutic strategy for the control of β -cell function in diabetes.

Here, we confirm that while visfatin is expressed in several adipose tissue depots, it is preferentially expressed in brown adipocytes. However, in rodents sympathetic activation *in vivo* does not reveal a positive correlation between visfatin expression and BAT activation. It is now well established that brown-like adipocytes appear in white adipose tissue after chronic sympathetic stimulation. These brite, or beige, adipocytes are found preferentially in subcutaneous white adipose tissue depots, where they form clusters of thermogenic competent cells able to burn lipids and glucose ¹⁰. Our results demonstrate that visfatin is highly expressed and secreted in genuine brown compared to white adipocytes, but not in brite adipocytes derived from scWAT progenitors.

Surprisingly, visfatin mRNA expression was found up-regulated in mouse eWAT upon cold exposure or CL316,243 treatment. This is not related to brite adipocytes recruitment, as eWAT is less sensitive to these treatments and does not display high amounts of UCP1 positive cells. However, eWAT is a highly lipolytic tissue under the control of adrenergic stimulation. Therefore, we can assume that visfatin expression could be related rather to the lipolytic function of white adipocytes.

As BAT found in adult humans correspond more to the inducible brite adipocytes described in rodents ²³, it is not surprising to find comparable levels of visfatin mRNA in BAT and WAT biopsies from adult volunteers. Similar observations are obtained *in vitro* using primary cultures of cells from scWAT and differentiated in white or brite/brown adipocytes.

Brite adipocytes originate from either specific white adipose tissue resident progenitors or from the conversion of mature white adipocytes ¹¹. Using hMADS cell models of white to brite adipocyte conversion, we show that visfatin mRNA expression is slightly increased during the conversion of white to brite adipocytes. Furthermore, this increase in mRNA expression is not correlated to an enhanced secretion of visfatin protein. As visfatin has been described to be a PPAR γ target gene in macrophages ²⁴, and as white to brite conversion *in vitro* is triggered by the addition of the PPAR γ agonist, rosiglitazone, it is tempting to suggest that this increased visfatin expression is due to PPAR γ activation.

Conflicting data have been reported for visfatin plasma levels in various cohorts of diabetic and obese patients as well as in rodents. Unexpectedly, several studies described a positive correlation between plasma visfatin levels and obesity, T2D and metabolic syndrome, situations associated *in fine* to β -cell dysfunction ²⁵⁻²⁷. Other studies did not reveal any association between visfatin circulating levels and visceral fat mass or insulin sensitivity parameters in humans and rodents ^{28, 29}. Altogether, these data highlight a paradox between pathologic visfatin circulating levels and its positive involvement in β -cell function.

In conclusion, visfatin can be considered as a rodent brown adipocyte biomarker. Nevertheless, its expression and thus secretion does not appear related to brown adipocyte activation or brite adipocyte recruitment in humans and rodents. While the currently available data do not permit to herald visfatin as a beneficial BAT-derived cytokine for the treatment of

obesity and T2D, the search for other potential brown/brite adipocyte secreted molecules, acting in concert or not with visfatin, remains a burning issue.

Material and methods.

Cell culture.

Mouse primary adipocytes. Animals were euthanized by cervical dislocation for preparation of stromal vascular fractions. Interscapular, perigonadal and subcutaneous fat depots were rapidly excised, washed in PBS, and minced. Adipose tissue samples were then digested with mild agitation for 45 min at 37°C in DMEM (Lonza, BE12-707F) containing 2 mg/ml collagenase A (Roche Diagnostics, 11088793011) and 20 mg/ml BSA (Sigma-Aldrich Chemie GmbH, A7030). Digested tissues were filtrated through 250, 100 and 27 µm nylon sheets, and centrifuged for 5 min at 500 g. Adipocyte fractions were obtained after centrifugation of the first 250 µm filtration at 500 g for 5 min, floating adipocytes were sampled for molecular analysis. The pellet containing stromal-vascular fraction (SVF) cells was submitted to red blood cell lysis procedure. SVF cells were either used directly for molecular analysis or plated and maintained in DMEM containing 10% (v/v) FCS until confluence. Differentiation was induced in the same medium supplemented with 1 µM dexamethasone (Sigma-Aldrich Chemie GmbH, D4902), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich Chemie GmbH, I5879) and 860 nM insulin (Invitrogen, 12585014) for 2 days. Cells were then maintained for 7-10 days in the presence of 10 nM insulin for white adipogenesis or a mixture containing 10 nM insulin, 1 µM rosiglitazone (BertinPharma, 71740) and 0.2 nM triiodothyronine (Sigma-Aldrich Chemie GmbH, T6397) for brown **or brite** adipogenesis.

hMADS and hADSC. The establishment and characterization of the multipotency and self-renewal capacity of hMADS cells have been described ³⁰. White and brite adipocyte differentiation were performed as previously described ³¹⁻³³. For human adipose-derived stem cells (hADSC), subcutaneous adipose tissue samples were obtained as *res nullus* from surgeries

(non-pathologic abdominoplasty). hADSC cells were isolated by enzymatic digestion as described in the mouse primary adipocytes section. Cells were then plated and maintained in culture using the protocol used for hMADS cells.

Isolation and analysis of RNA.

Total RNA was extracted using a TRI-Reagent kit according to manufacturer's instructions (Euromedex, RT-111), with an additional step for tissues consisting to homogenization in TRI-Reagent using an ULTRA TURRAX T25 (Ika). Reverse transcription and polymerase chain reaction (RT-PCR) analysis were performed as described previously³³. The oligonucleotide sequences are shown in Table 1. Quantitative PCR (qPCR) was performed using SYBR qPCR premix Ex TaqII from Takara (Ozyme, TAKRR820W) and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life and Analytical Sciences). The expression of selected genes was normalized to that of 36B4 housekeeping genes and then quantified using the comparative- ΔC_t method.

Visfatin secretion assay.

Differentiated SVF from mouse adipose tissue or differentiated hMADS cells were incubated for 16h in fresh media (serum free, BSA 0.05%) stimulated or not with 1 μ M CL316,243. Conditioned media were sampled and centrifuged for 5 min at 400 g to remove cell debris. Attached cells were lysed in a buffer containing 25 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P40, 1x protease inhibitor cocktail (Roche, 11697498001). **Visfatin levels were quantified in cell lysates and conditioned media using specific ELISA kit from Enzo Life Sciences for mouse (AG-45A-0007EK-KI01) or RayBiotech**

for human samples (Clinisciences, EIA-VIS-1). Assays were performed following manufacturer's instruction.

Animals.

The experiments were conducted in accordance with the French and European regulations for the care and use of research animals and were approved by the local experimentation committee CIEPAL-Azur N°28 (protocol NCE-2012-57, NCE-2014-166 and MESR-00500.02). Animals were maintained under constant temperature (mice: $21 \pm 2^{\circ}\text{C}$; rats: $23 \pm 2^{\circ}\text{C}$) and 12:12-hour light-dark cycles, with *ad libitum* access to standard chow diet and water. C57Bl/6J mice (males, 10 to 14 weeks-old) and Wistar rats (males, 12 to 16 weeks-old) were from Janvier Labs (<http://www.janvier-labs.com/>). Brown adipocyte activation was induced by a daily intra-peritoneal injection of a $\beta 3$ -adrenergic receptor agonist CL316,243 (1 mg/kg in a saline solution, Sigma-Aldrich Chemie GmbH, C5976) for 7 days. Control animals were injected with vehicle only. For cold exposure, mice were exposed for 4 days and rats for one night at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a climate chamber.

Subjects.

The study protocol was reviewed and approved by the ethics committee of the Hospital District of Southwestern Finland, and subjects provided written informed consent following the committee's instructions. The study was conducted according to the principles of the Declaration of Helsinki. All potential subjects were screened for metabolic status, and only those with normal glucose tolerance and normal cardiovascular status (as assessed on the basis of electrocardiograms and measured blood pressure) were included. The age range of the subjects was 23-49 years. We studied a group of six healthy volunteers (2 men and 4 women). BAT was

sampled in positive FDG-PET scan site, and subcutaneous WAT was derived via the same incision.

Statistical analyses.

Data are expressed as mean values \pm SEM and were analyzed using InStat software (GraphPad Software). Data were analyzed by one-way ANOVA followed by a Student-Newman-Keuls post-test, or Student's t-test to assess statistical differences between experimental groups. Differences were considered statistically significant when $p < 0.05$.

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Figure Legends

Figure 1. Visfatin expression in mouse brown and white adipocytes and stromal vascular fractions. mRNA expression determined by RT-qPCR in Stroma Vascular Fraction (SVF) and adipocyte fraction (AF) from scWAT, eWAT and iBAT from C57BL/6 mice. **Perilipin-1 was used as control of adipocyte purification, and leptin as a white adipocyte marker.** Histograms represent mean±sem of 8 mice. *: $p<0.05$.

Figure 2. Visfatin expression in mouse brown and white adipocytes. (A) Cells from SVF of the three adipose tissue depots were differentiated in white or brown adipocytes and used for mRNA level evaluation by RT-qPCR. **Perilipin-1 was used as control of adipogenesis, and leptin as white adipocyte marker.** (B) Intracellular and extracellular visfatin protein levels were quantified by EIA in white and brown adipocytes, derived from SVF of the three adipose tissue depots, after 16 h of incubation in the absence or presence of 1 μ M CL316,243. Histograms represent mean±sem of 3 independent experiments (B). *: $p<0.05$.

Figure 3. Visfatin is preferentially expressed in mouse BAT, but is not correlated to brown adipocyte recruitment/activation. mRNA expression was determined by RT-qPCR in iBAT, scWAT and eWAT from C57BL/6 mice exposed (4 days) at a temperature of $4\pm 2^{\circ}\text{C}$ (A) or received CL316,243 for 1 week (B). Control animals were respectively maintained at housing temperature ($21\pm 2^{\circ}\text{C}$) or injected with the vehicle (NaCl 0.9%, w/v). **Perilipin-1 was used as a control marker of adipose tissue.** Histograms represent mean±sem of 8 mice. *: $p<0.05$.

Figure 4. Visfatin is preferentially expressed in rat iBAT, but is not induced with BAT activation. mRNA expression was determined by RT-qPCR in iBAT and eWAT from Wistar

rats exposed overnight at a temperature of $4\pm 2^{\circ}\text{C}$ (A) or received CL316,243 for 1 week (B). Control animals were maintained near to rat thermoneutral temperature ($23\pm 2^{\circ}\text{C}$) or injected with the vehicle (NaCl 0.9%, w/v). **Perilipin-1 was used as a control marker of adipose tissue.** Histograms represent mean \pm sem of 4 rats. *: $p<0.05$.

Figure 5. Visfatin expression and secretion in human white and brown adipocytes. (A) mRNA levels were evaluated by qRT-PCR in matched biopsies of scWAT and BAT from 6 healthy human adult patients. mRNA expression determined in human subcutaneous adipose tissue SVF-derived white and brown adipocytes (B) or hMADS adipocytes (C). **Perilipin-1 was used as control of adipogenesis and leptin as white adipogenic marker.** (D) Intracellular and extracellular visfatin EIA quantification in hMADS white and brown adipocytes after 16h of incubation with or without $1\mu\text{M}$ CL316,243. Histograms represent mean \pm sem of 6 tissue samples (A) or 3 independent experiments (B, C, D). *: $p<0.05$.

Table 1. Sequence of primers used for gene expression analysis.

Genes	Species	Forward primer	Reverse Primer
Visfatin	human	AGCCGAGTTCAACATCCTCC	AACTTTGCTTGTGTTGGGTGG
UCP1	human	CCAGGATCCAAGTCGCAAGA	GTGTGCCCAACTGTGCAATG
perilipin 1	human	ACCCCCCTGAAAAGATTGCTT	GATGGGAACGCTGATGCTGTT
leptin	human	AGGGAGACCGAGCGCTTTC	TGCATCTCCACACACCAAACC
36B4	human	TGCATCAGTACCCCATCTATCAT	AGGCAGATGGATCAGCCAAGA
Visfatin	rat	TCTGGAAATCCGCTCGACAC	TATCCACTCCGTCCCCTTGA
UCP1	rat	TGGCCAAGACAGAAGGATTG	GATCTTGCTTCCCAAAGAGG
perilipin 1	rat	TCTGCTTTGCAGCGTGAAGA	GGTCTTCTGGAAGCACTCACA
36B4	rat	ATCGTCTTTAAACCCCGCGT	CCACATTGTCTGCTCCCACA
Visfatin	mouse	ATTCAAGGAGATGGCGTGGA	CTTCTGTAGCAAAGCGCCAC
UCP1	mouse	CACCTTCCCGCTGGACACT	CCTGGCCTTCACCTTGGAT
perilipin 1	mouse	AGCGTGGAGAGTAAGGATGTC	CTTCTGGAAGCACTCACAGG
leptin	mouse	GCTGGTGAGGACCTGTTGAT	CAGGATCAATGACATTTACACA
36B4	mouse	TCCAGGCTTTGGGCATCA	CTTTATCAGCTGCACATCACTCAGA

Figure 1.

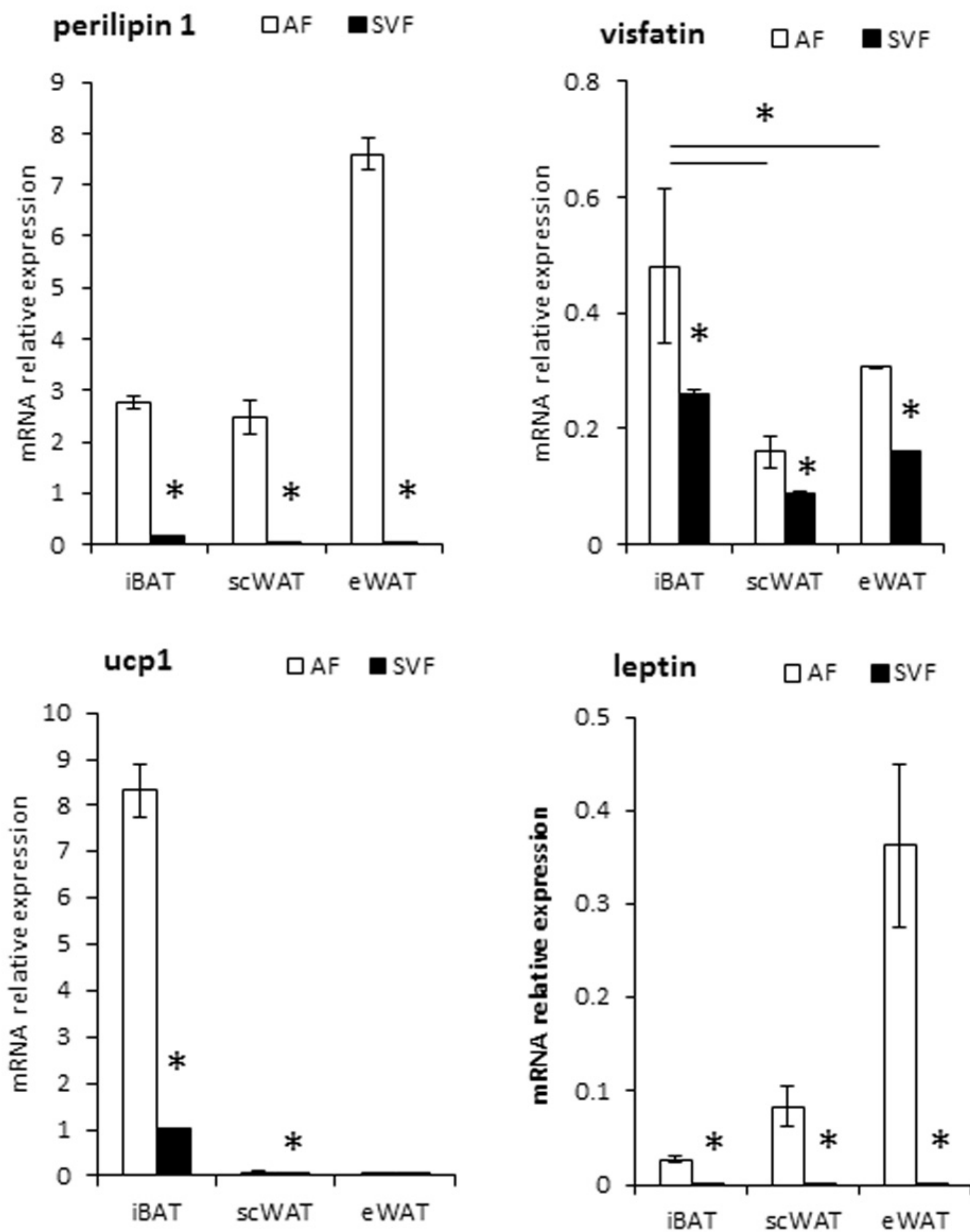


Figure 2.

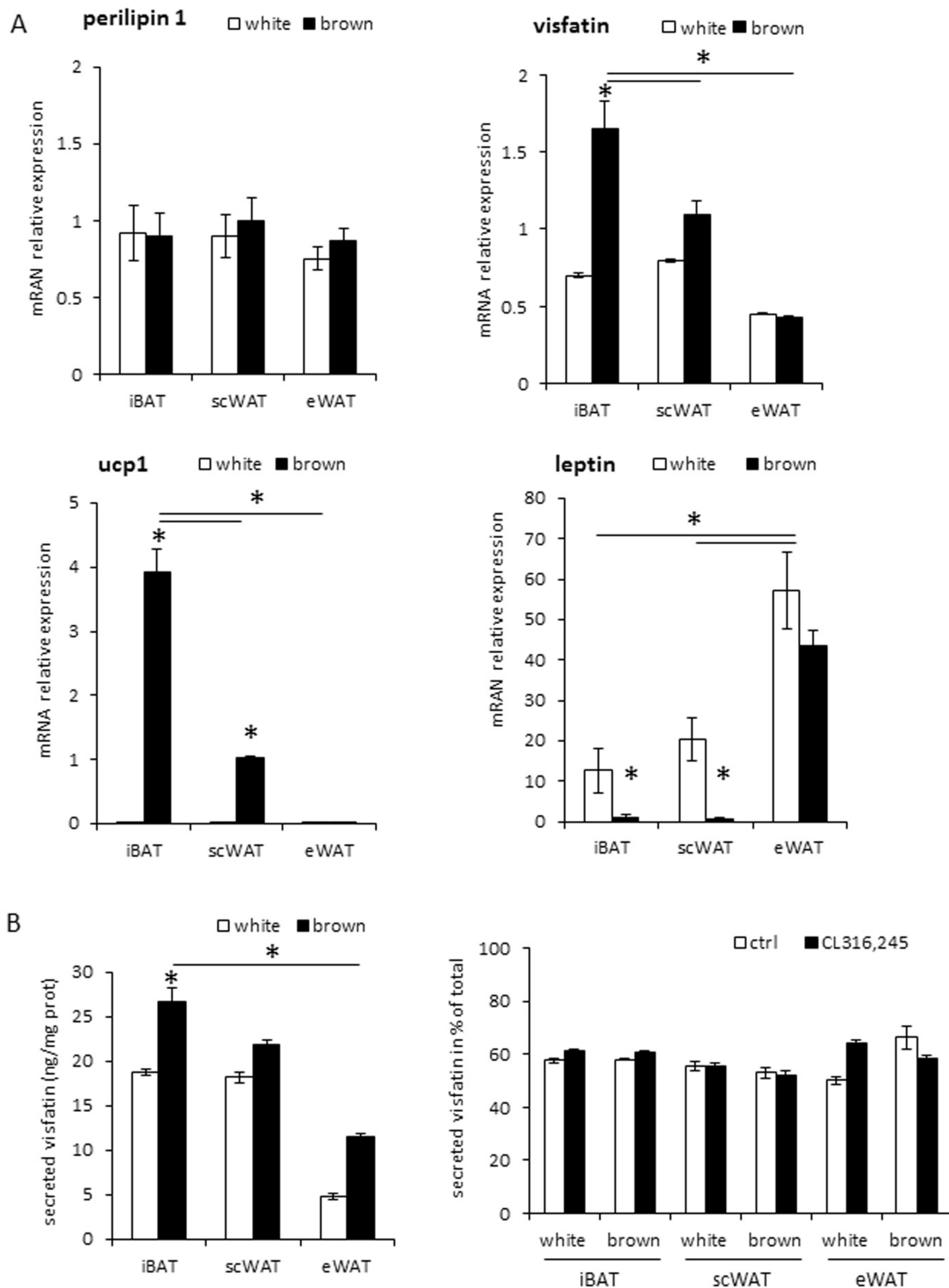
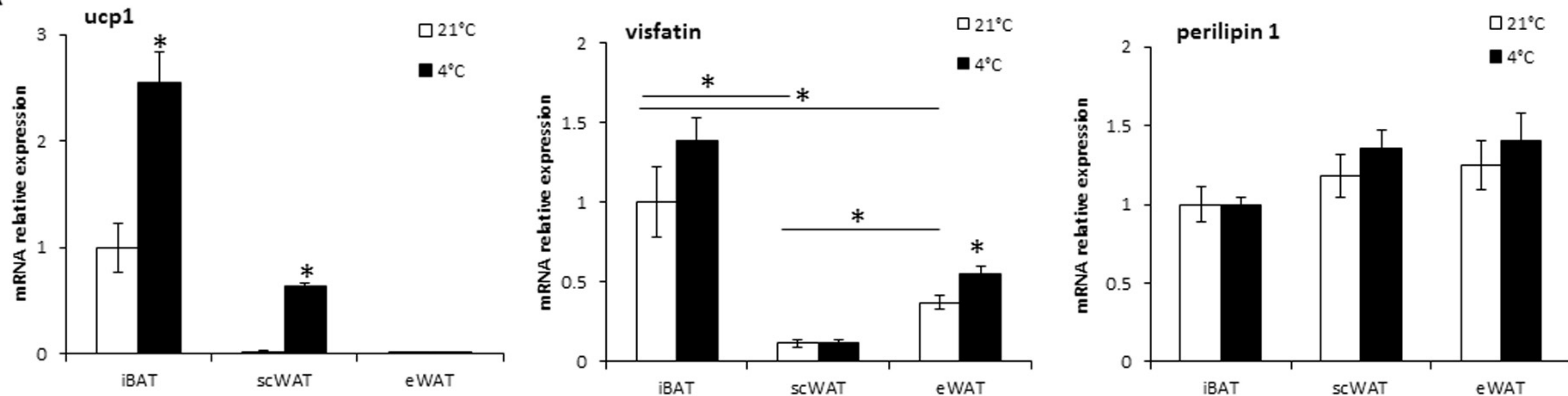


Figure 3.

A



B

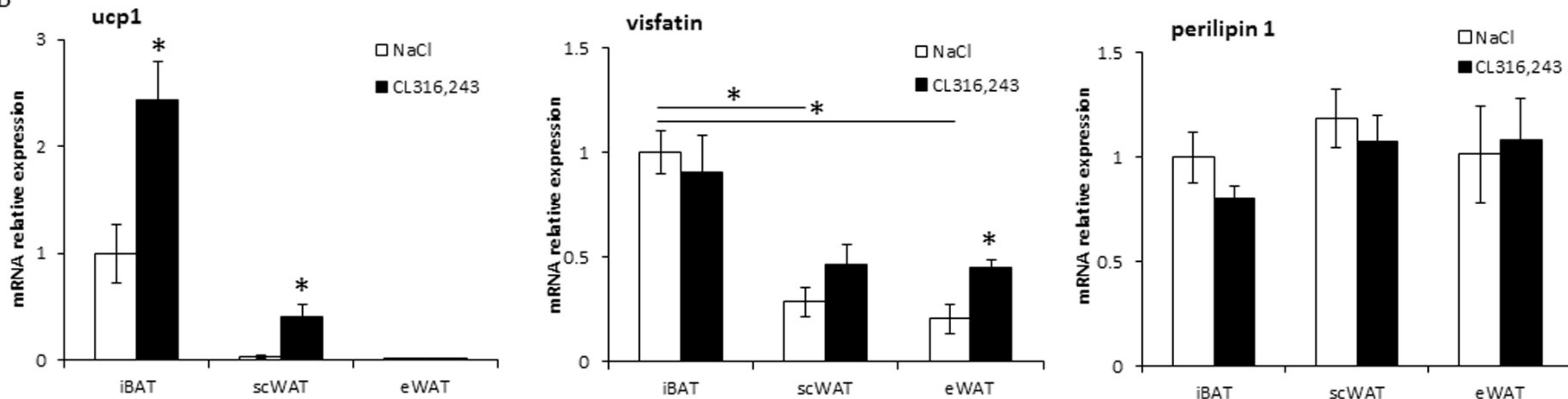


Figure 4.

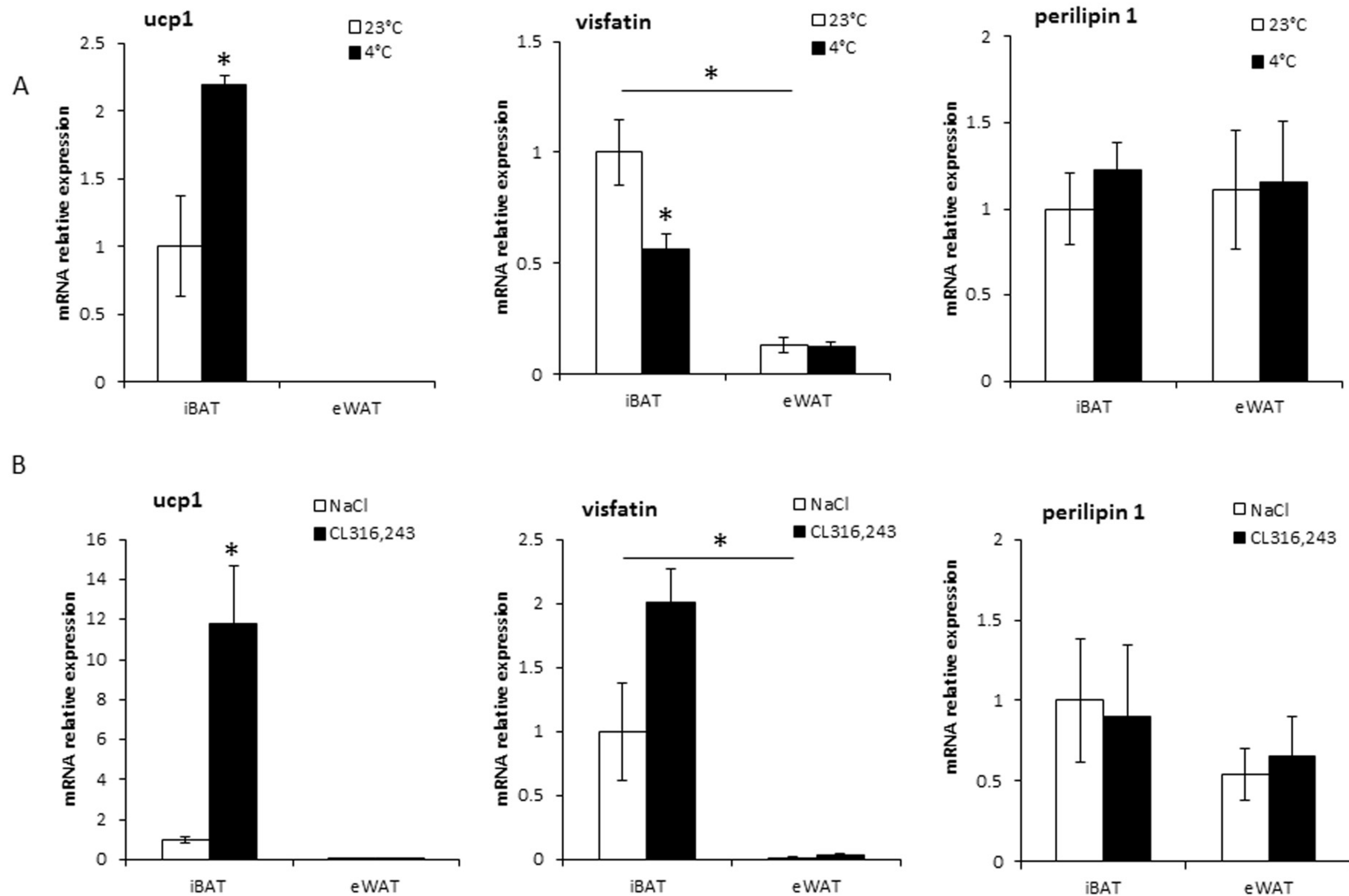


Figure 5.

