Increased expression level of ANGPTL8 in White Adipose tissue under Chronic Cold Treatment

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Research Article

Keywords: Angiopoietin like proteins, ANGPTL, Adipose Tissue, Browning, Cold Treatment, UCP1

DOI: https://doi.org/10.21203/rs.3.rs-512132/v1

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Abstract

Background

It is well known that Angiopoietin like protein (ANGPTL) mainly 3, 4 and 8 play a major role in maintaining energy homeostasis by regulating lipoprotein lipase (LPL) activity, which is a key regulator of triglyceride (TG) metabolism. Our aim is to evaluate the level of ANGPTL3, 4 and 8 in mice maintained under cold conditions in the liver, brown adipose tissue (BAT), and white adipose tissue (WAT).

Methods

C57BL/6J mice were exposed to cold temperature at 4°C over a period of ten days with food given ad libitum. Animal tissues were harvested at days 0, 1, 3, 5, and 10 (cold treatment groups, n = 10 in each group, control, n = 5). Liver, subcutaneous adipose tissue (SAT), and BAT were used to investigate the expression level of different genes. ANGPTL3, 4 and 8 genes expression were measured in liver. ANGPTL4, 8 and UCP1 were measured in SAT and BAT.

Results

ANGPTL 3 and 8 gene expression levels were significantly reduced in mice liver tissues after cold treatment (P-value < 0.05). However, the gene expression level of ANGPTL4 was not significantly changed. In BAT, ANGPTL8 expression was not changed after cold treatment while ANGPTL4 was significantly reduced (P-value < 0.05). ANGPTL4 level was also significantly reduced in SAT, whereas the gene expression level of ANGPTL8 showed over a 5-fold increase. Similarly, the UCP1 gene expression was also significantly increased in SAT. Additionally, protein overexpression of ANGPTL8 was further confirmed by immunohistochemistry after extended cold treatment.

Conclusion

Our data shows that ANGPTL proteins are inhibited in the liver and BAT under cold treatment. This agrees with other studies that showed that reduction in ANGPTL4 in BAT improved thermogenesis in response to acute cold exposure. However, in our study we also observed that ANGPTL8 is activated under these conditions in SAT. This suggests that it might be involved in the regulation of lipolysis as well as enhancing SAT browning.

Introduction

Lipid metabolism constitute an important energy source which is often dysregulated in metabolic diseases such as obesity and type 2 diabetes mellitus [1]. Triglycerides (TG) constitute an important energy source that are present in all lipoprotein classes and are highly enriched in chylomicrons and very
low-density lipoprotein (VLDL) [2]. Chylomicrons transfer dietary fat from the intestine to peripheral tissues while VLDL are synthesized in the liver [2]. They both act as carriers of triglycerides which undergo lipolysis by the lipoprotein lipase (LPL) enzyme intravascularly to release free fatty acids that are either used by peripheral tissues such as muscles as an energy source or stored in adipocytes as fat [3-5]. Triglyceride partitioning and metabolism is a highly regulated process by several proteins that regulate LPL activity, mainly members of the Angiopoietin like protein (ANGPTL) family as well as others such as apolipoprotein CIII. Angiopoietin like proteins 3, 4 and 8 have been shown to directly regulate LPL activity [3-5].

ANGPTL proteins are either exclusively expressed in the liver such as ANGPTL3 or expressed by the liver, adipocytes, and other tissues such as ANGPTL4 and 8 [5-8]. A well accepted ANGPTL3-4-8 model of the role of ANGPTL proteins postulates that these three proteins regulate LPL activity in a tissue specific manner depending on the nutritional status [8]. While ANGPTL3 is stable, ANGPTL4 and 8 respond differently to food cues where ANGPTL4 is induced by fasting [8]. This induction leads to the inhibition of LPL activity in WAT directing TG to cardiac and skeletal muscles and away from fat storage. On the other hand, feeding induces ANGPTL8 expression and the formation of ANGPTL3/ANGPTL8 complex that inhibits LPL activity in cardiac and skeletal muscles directing TG to storage in adipocytes [8]. This model is further refined by the new finding showing that ANGPTL8 is also capable of interacting with ANGPTL4 forming an ANGPTL4/ANGPTL8 complex that acts in an opposite fashion to ANGPTL3/ANGPTL8 complex ensuring that LPL is not inhibited at the adipocytes after feeding [9]. Thus, the increased ANGPTL8 expression after feeding acts in opposite fashion depending on its dimerization with ANGPTL3 or ANGPTL4. In skeletal muscles, ANGPTL8 binds to ANGPTL3 and inhibit LPL while in adipocytes it forms a complex with ANGPTL4 leading to increased LPL activity directing postprandial fatty acid to adipose tissue for storage [9].

In addition to its role in regulating lipid metabolism in circulation, an intracellular role of ANGPTL8 in lipid metabolism has been proposed in adipocyte differentiation [10]. This role has been mostly inferred based on its increased expression during adipocyte differentiation [10]. One of the most important organs involved in controlling lipid metabolism are the white and brown adipose tissues [11-13]. In addition to their role in energy storage, adipocytes release various signals that regulate whole body metabolism [11-13]. As a result, they were suggested to play a key role in the development of obesity and its associated metabolic abnormalities. On the other hand, brown adipocytes are a powerhouse that consumes a large amount of energy which can enhance energy expenditure and alleviates insulin resistance by creating a negative energy balance [11-13]. Brown adipose tissue facilitates the mitochondrial heat production through a non-shivering mechanism that uncouples ATP production from substrate metabolism [11, 14]. Moreover, shifting white adipocytes into brown or beige adipocytes is a field of high value as it constitutes a mechanism to increase energy expenditure and enhance metabolic function. Adipocyte browning is a natural process that is induced by cold treatment or by exercise [11, 14]. LPL is one enzyme that has been shown to be activated by short cold exposure [15]. This increased activity was attributed to the increased demand for fatty acid for thermogenesis [15]. Additionally, ANGPTL4 plasma level was shown to be increased after short-term cold treatment that placed individuals in a cold bath and gradually
lowered the water temperature until just above the shivering point of the participant [16]. This study only looked at the plasma levels of these markers for obvious limitations. To address the role of these proteins in the intracellular lipid metabolism, our study will evaluate the tissue specific expression of ANGPTL3, 4 and 8 in the liver, white adipose tissue (WAT) and brown adipose tissue (BAT) after an extended cold exposure to highlight their potential role in chronic cold adaptation.

Methods

Animal Studies and Cold Exposure

C57BL/6J mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA), bred and kept in a barrier animal facility in the Dasman Diabetes Institute (DDI) under constant temperature (22°C±1°C) and humidity in a 12 h controlled dark/light cycle (lights on: 7:00 am to 7:00 pm). All mice were kept with ad libitum access to a normal chew diet containing 6.2% calories as total fat (EURodent Diet 14%, 5LF2, LabDiet, St. Louis, MO, USA) and drinking water.

A total number of 45 male mice, age 12 weeks old were used in this study. Mice were individually kept in cages with minimum required bedding with free access to the food and water. The mice were randomly allocated in two groups, namely cold exposure group (n= 40) and room temperature control group (n=5). At the end of study, the levels of weight gain and food intake were measured, and the mice were sacrificed. Liver biopsies and fat depots including subcutaneous adipose tissue (SAT) and brown adipose tissue (BAT) were collected for further analysis. All the procedures were approved by the DDI Animal Care and Ethics Committee according to the international regulations and laws on animal research.

For cold exposure, 40 male mice were singly housed in prechilled cages with minimum bedding on a 12-hour dark/light cycle with ad libitum access to chow diet and water in a cold controlled environment at 4°C. At days 1, 3, 5, and 10 post-cold induction, mice (n=10 in each group) were sacrificed and the required samples were harvested for further analysis.

In a preliminary experiment, 10 mice were kept under cold conditions at the mentioned time points and their physical activity was observed and rectal temperature was monitored using a microprobe (50-7221F, Harvard Apparatus, Holliston, MA, USA) to rule out stress and hypothermia incidences.

RNA isolation and quantitative real-time PCR

Dissected tissues were immediately placed in RNAlater RNA Stabilization Reagent (Qiagen Inc.) and stored at −80°C for subsequent RNA extraction. Total RNA was extracted and isolated from the tissues using TRIzol reagent (Cat. No. 15596026, Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. For analyzing mRNA expression, the extracted samples were first quantified to assess their quality and concentration using the Epoch microplate spectrophotometer (Biotek Instruments, Inc.,
cDNA was synthesized from 1 μg RNA using the High-Capacity cDNA Reverse Transcription kit (Cat. No. 4368814, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in duplicates using SYBR Green primers and on an Applied Biosystems 7500 Real-Time PCR System. Relative gene expression (normalized to 18S) was calculated using the comparative CT method formula $2^{-\Delta \Delta CT}$ and reported as mean fold change in gene expression. The following primers were used for amplification: ANGPTL3 (FW: TGCACCTTCAGAGCCAAAAT, RV: CATTGGTTCGAAGTGATAGGTCA), ANGPTL4 (FW: ACAGTGACTTTGTTGTGGC, RV: CTCTAGCCCCATGGTTTCTGG), ANGPTL8 (FW: CTCTCCTCCTGTGGAC, RV: GCTCTGTACACGCCATTGAG), UCP1 (FW: CTTTGCTCAGTCAGTTGG, RV: ACTGCC ACACCTCCAGTCATT) and 18S (FW: CTGAGAAACGGCTACCACATC, RV: GGCCTCGAAAGAGTCCTGTAT).

Immunohistochemistry and Confocal imaging

Formalin fixed and paraffin embedded sections (of 8µm thickness) from SAT of each time point were used. These sections were de-paraffinized, rehydrated and antigen retrieved at pH6 using DAKO reagents (Dako, Glostrup, Denmark). Quenching of endogenous peroxidase activity was done using 3% hydrogen peroxide solution (1 hr at room temperature). The sections were then blocked, first with 5% fat free milk followed by 1% BSA. They were then incubated with the primary antibody ANGPTL 8 (MAB8548, R&D systems, USA) at 4˚C, overnight. Alexa fluor 488 conjugated secondary antibody was used at RT for 1 hr (A-11008, Invitrogen, USA, 1:100 dilution). Nuclear staining was done using DAPI at 0.05%. Zeiss LSM 710 confocal laser scanning microscope (Zeiss, Germany) was used to acquire the fluorescence images. The images were taken at 40X magnification for each group. Zen software (Zeiss, Germany) was utilized for quantification of the image intensities.

Statistical analysis

The minimum sample size of 4-6 mice per group was estimated to obtain statistical significance, considering the error = 0.05, power = 0.80, percentage change in means (PC) = 20%, and co-efficient of variation (CV) = 10 ~ 15% (varies between the experiments). Together with our prior experience, we used a total of 5 mice as a room temperature control group, and n=10 mice in each group for cold exposure to ensure adequate power. Values are expressed as means of duplicate experiments ± SEM of each harvested sample. All data was analyzed, and figures were prepared using GraphPad Prism 6 (GraphPad Software). Statistical differences between two groups were assessed by unpaired, 2-tailed Student's t test and between multiple groups using nonparametric Kruskal–Wallis test for multiple comparisons. A $P$ value of less than 0.05 was considered statistically significant.

Results

Animal weight and food Consumption
Body weight and food intake was measured for each of the mice at the specific time points, D0, D1, D3, D5 and D10. No significant change in average body weight was observed between mice at the various time points (Figure 1A). A significant increase in food consumption was detected following cold exposure for several days particularly among mice at D3 when compared to control mice (Figure 1B). Interestingly, measuring the weight of total SAT and BAT collected from mice in the study showed a significant decrease in the average SAT weight across the different time points of cold exposure (Figure 1C, p<0.05). On the other hand, a significant increase in the average BAT weight was observed in mice exposed to cold over time, with highest increase in weight observed at D10 (Figure 1D, p<0.05).

 Liver ANGPTL Gene Expression

Gene expression analysis of ANGPTL genes of interest (ANGPTL3,4 and 8) was performed on mRNA extracted from liver biopsies. Results obtained showed a significant decrease in the expression levels of ANGPTL3 and ANGPTL8 (0.2-fold change and a 0.5-fold change respectively) (Figure 2A, 2C, p<0.05). No significant change in expression level of ANGPTL4 was observed (Figure 2B).

 Adipose tissue (BAT and SAT) ANGPTL and UCP1 Gene Expression

Gene expression analysis was also performed on mRNA extracted from SAT and BAT biopsies. The expression level of UCP-1, which is a gene known to play an important role in the process of adipose tissue browning, was significantly increased in SAT across various time points of cold exposure particularly on D3 with a 20-fold increase in gene expression levels was observed when compared to D0 (Figure 3A, p<0.0001). However, in BAT a significant increase was seen at D1 with a 3.2-fold increase when compared to D0 of cold exposure (Figure 4A, p<0.05). A decrease in UCP-1 expression to normal levels was observed in BAT over time in cold (Figure 4A). Analysis of gene expression level of ANGPTL 4 and 8 in mRNA extracted from SAT biopsies showed a decrease in the level of ANGPTL4 over time in cold with most significant decrease seen at D5 and D10 of cold exposure with a 0.5-fold change and a 0.3-fold change, respectively (Figure 3B, p<0.001). On the other hand, a significant increase was observed in ANGPTL8 expression level in SAT with highest increase seen at D1 of cold exposure with a 7.2-fold increase when compared to D0(Figure 3C, p<0.001). Looking at the gene expression levels of ANGPTL4 and 8 in BAT we observed a significant decrease in ANGPTL4 level starting at D1 of cold exposure (0.5-fold change, Figure 4B, p<0.001).No change in gene expression level of ANGPTL8 was detected in BAT at any time point of cold exposure (Figure 4C).

 SAT ANGPTL8 Protein Expression

To confirm the gene expression data obtained from SAT, we performed protein expression analysis using IHC. The results obtained show a significant increase in the fluorescence intensity in SAT sections from tissues extracted from mice exposed to cold treatment when compared to SAT sections obtained from tissues of control mice (Figure 5). The results indicate an increase in the protein expression of ANGPTL8 in SAT with chronic cold treatment.
Discussion

In this study we explored the impact of chronic cold treatment on the tissue expression of three members of the ANGPTL family, ANGPTL3, 4 and 8. In circulation, these members are known to play a major role in regulating the activity of LPL, an important enzyme in triglyceride metabolism. In the liver, ANGPTL3, and 8 were inhibited after short cold exposure and this inhibition was maintained throughout the course of the ten-day treatment while ANGPTL4 was not affected by the cold treatment. In SAT, UCP1 expression was dramatically induced after cold treatment and this induction was also maintained throughout the course of the treatment. ANGPTL8 showed a similar pattern on increased expression after cold treatment in SAT as well while ANGPTL4 was reduced. Since ANGPTL3 is exclusively expressed in the liver, its expression was not tested in the other tissues. In BAT, UCP1 was significantly induced after 24hrs of cold treatment but then maintained its expression level. ANGPTL4 on the other hand was significantly reduced while ANGPTL8 was not changed during treatment. Collectively, these data show that ANGPTL proteins are mainly reduced or unaffected during cold treatment except for ANGPTL8 in SAT is dramatically increased suggesting that it might be involved in the regulation of lipolysis as well as enhancing SAT browning.

Triglyceride metabolism is an important energy source of energy for BAT through the LPL induced lipolysis of triglyceride rich lipoproteins [17-20]. Under cold treatment, BAT generates heat to maintain body homeostasis breaking down intracellular lipids which is also replenished by the action of LPL breakdown of triglycerides to fatty acids. Cold treatment initiates this process by the activation of sympathetic β-adrenergic receptors in BAT tissues [17-20]. Intracellularly, triglycerides stored in lipid droplets are released as fatty acids through the activity of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) [21, 22]. Once in the mitochondria, fatty acids lead to activation of UCP1 which uncouples the proton gradient from ATP production to heat generation.

It is well established that the main source of energy in this process is triglyceride lipolysis and not direct fatty acid uptake as demonstrated by the impaired thermogenesis in BAT lacking the key lipolysis protein ATGL [21, 22]. Extracellularly, LPL activity is increased at BAT directing fatty acids produced from TG breakdown toward BAT. It has been shown that LPL activity is increased at the post-translational level and not transcription [3, 15]. Reduction in ANGPTL4 has also been shown to be lowered in extended cold exposure releasing its inhibition of LPL activity in BAT [18]. This also demonstrates the role of maintaining LPL activity to replenish the intercellular storage of triglycerides. As a result, inhibition of ANGPTL4 is important to maintain the LPL activity [18]. However, in our study we did not see a decrease in liver ANGPTL4 level but rather a decrease in ANGPTL8 level. This can be explained by the fact that ANGPTL8 mediated LPL inhibition has been shown to function in complex with either ANGPTL3 or ANGPTL4. As a complex ANGPTL8/ANGPTL3 or ANGPTL8/ANGPTL4 can be either 200-fold inhibitory or 125-fold less inhibitory towards LPL compared to ANGPTL3 or ANGPTL4 alone, respectively [9]. As a result, further analysis of the protein complexes might be necessary to highlight this difference in gene expression.

Liver plays a major role in energy homeostasis through regulating glucose and lipid metabolism pathways. It’s the major source of ANGPTL3 and ANGPTL8 which are well known regulators of LPL.
activity [4, 23-26]. LPL is expressed by multiple tissues such as adipose tissues, heart, and skeletal muscles amongst others [4]. Both TG-rich VLDL and chylomicrons are hydrolyzed by LPL lumen of the capillaries releasing free fatty acids and monoacylglycerol that is absorbed by peripheral tissues for storage in the form of TG or oxidation for energy generation [4]. LPL activity is regulated by the cycle of feeding and fasting through ANGPTL3, 4 and 8 proteins [4]. After feeding, ANGPTL3 in combination with ANGPTL8 act as a potent inhibitor of LPL activity to direct triglyceride rich particle to white adipocytes for storage [8]. On the other hand, during fasting, ANGPTL4 inhibits LPL activity in adipose tissue directing LPL activity to oxidative tissues [8]. Extended cold treatment will exert a high demand for energy conversion to heat in the brown adipocytes. As a result, it is expected that ANGPTL3 and ANGPTL8 to be inhibited in the liver to maintain a high LPL activity and direction of FA for BAT tissue for heat generation. ANGPTL4 was also expected to be decreased but its liver expression was maintained throughout the cold treatment. This could be explained by the fact that ANGPTL4 is induced mainly by fasting and ANGPTL4 level was not expressed that high to start since the animals were in the fed state ad libitum. On the other hand, circulating level of ANGPTL8 has been suggested to be from hepatocytes as demonstrated by Vatner et al., [27]. It has also been suggested that ANGPTL8 affects intracellular lipid metabolism though an LPL independent mechanism. This can be evident in the paradoxical observation that ANGPTL8 knockout have no impact on circulating TG level but cause a decrease in TG level in the fed estate [4]. Additionally, it has been demonstrated that treating 3T3-L1 cells with recombinant ANGPTL8 is capable of inhibiting lipolysis through inhibition of ATGL and HSL which was also reported in HepG2 cells [4, 8]. As a result, reduction of ANGPTL8 hepatocyte levels in our chronic cold treatment might have a direct impact increasing ATGL activity ensuring more energy is directed toward thermogenesis.

Amongst the many roles WAT plays in physiology, its role as an energy storage under cold treatment is very critical in directing lipids toward BAT for thermogenesis. However, WAT is a very dynamic tissue. Adipose tissue response to cold demonstrates the elasticity and the highly dynamic state this tissue possesses [11, 14, 19]. This is clearly shown in the WAT tissue response to extended cold treatment where it can be differentiated into what is called beige or brite adipocytes [11, 14, 19]. Like BAT, beige cells exhibit a high level of UCP1 and many mitochondria with high thermogenetic capacity. Norepinephrine play a major role in this pathway which is released from sympathetic nerve acting on adipocytes β-adrenergic receptors. Irisin, meteorin-like, and fibroblast growth factor-21 (FGF21) are other identified factors that play an important role in enhancing the browning of white adipocytes [28-33]. Irisin has been shown to positively associate with ANGPTL8 after exenatide treatment of T2D patients. ANGPTL8 was also induced by irisin during adipocytes differentiation [34, 35]. In addition, ANGPTL8 has also been shown to be induced during adipocytes differentiation and more recently was shown to have a similar expression pattern like PPARγ, C/EBPα and LPL in the preadipocyte differentiation and adipogenesis [36]. The authors also concluded through a series of experiment involving knockdown and overexpression of ANGPTL8 that it can significantly promote lipid deposition and adipocyte differentiation [36]. In line with these observations, in this study WAT ANGPTL4 was significantly reduced after an extended cold treatment while ANGPTL8 was induced. This striking contrast in the gene expression of these two proteins highlight a potentially second role of ANGPTL8 in SAT. ANGPTL8 and
UCP1 followed a similar pattern of expression in SAT. This parallel increase suggests that ANGPTL8 might be involved in SAT beiging. This remains to be further validated in a tissue specific knockout model and further experimentation.

One of the limitations of this study is the design of the experiment to have food available throughout the cold treatment. Under these conditions non-shivering thermogenesis in BAT energy source can be either fatty acids or carbohydrates from food. Under fasting conditions, fatty acids will be the main energy source utilizing the ATGL mediated lipolysis. However, due to the extended cold treatment animals will not survive without food being available. In conclusion, our data shows that ANGPTL proteins are inhibited in the liver and BAT under cold treatment. This agrees with other studies that showed that reduction in ANGPTL4 in BAT improved thermogenesis in response to acute cold exposure. However, in our study we also observed that ANGPTL8 is activated under these conditions in SAT. This suggests that it might be involved in the regulation of lipolysis as well as enhancing SAT browning. Additionally, this study further highlights the complexity of the interaction between the ANGPTL proteins and their importance in regulating lipid metabolism during normal and stressful conditions.

Declarations

Competing Interests The authors declare that they have no competing interests

Abbreviations

ANGPTL: Angiopoietin-like protein
WAT: White Adipose Tissue
BAT: Brown Adipose Tissue
SAT: Subcutaneous Adipose Tissue
LPL: Lipoprotein Lipase
TG: Triglyceride
UCP1: Uncoupling Protein 1
VLDL: Very Low-Density Lipoprotein
CV: Coefficients of variation

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**Figures**

Figure 1

Animal Characteristics throughout cold treatment and food consumed. A: Animal weight over the ten days of cold treatment in grams. B: Amount of daily consumed food over the ten days period. C: SAT weight in mg over the ten days period. D: BAT weight in mg over the ten days period.
Figure 2

Liver ANGPTL3, ANGPTL4 and ANGPTL8 gene expression. A: Liver level of ANGPTL3 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. B: Liver level of ANGPTL4 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. C: Liver level of ANGPTL4 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. * p < 0.05, as determined using Student’s t-test.
Figure 3

SAT UCP1, ANGPTL4 and ANGPTL8 gene expression. A: SAT level of UCP1 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. B: SAT level of ANGPTL4 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. C: SAT level of ANGPTL8 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. * p < 0.05, as determined using Student’s t-test.
Figure 4

BAT UCP1, ANGPTL4 and ANGPTL8 gene expression. A: BAT level of UCP1 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. B: BAT level of ANGPTL4 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. C: BAT level of ANGPTL8 as measured by RT-PCR after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C. * p < 0.05, as determined using Student’s t-test.
**Figure 5**

Immunohistochemistry staining of SAT tissue to estimate the expression level of ANGPTL8 after a prolonged cold treatment. Gene expression was measured at Day 0, 1, 3, 5 and 10 days post cold treatment at 4°C.

**Supplementary Files**

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