Growth hormone inhibits adipogenic differentiation and induces browning in bovine subcutaneous adipocytes

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Abstract

It is well established that growth hormone (GH) has the ability to stimulate lipolysis. The effects of GH on adipocyte differentiation and browning have not been clearly described. Therefore, the present study aimed to elucidate the role of GH in the differentiation and browning of bovine subcutaneous adipocytes as well as its underlying molecular mechanisms. In this study, we demonstrated that GH inhibited lipid accumulation and decreased the expression levels of adipogenic key genes (SCD1, SREBP1, PPARγ, and CEBPα) during adipocyte differentiation. Moreover, we observed that the inhibitory effect of GH on the early stage of adipocyte differentiation (0–2 days) was stronger than that on the later stage of adipocyte differentiation (2–8 days). We also found that GH promoted the expression levels of browning-related genes such as uncoupling protein 1 (UCP1) in mature adipocytes. Concurrently, GH promoted mitochondrial biogenesis and increased the expression levels of mitochondrial biogenesis-related genes. In addition, GH promoted phosphorylation of signal transducers and activator of transcription 5 b (STAT5B) and contributed to translocation of STAT5B to nucleus. After blocking the expression of STAT5B protein, GH weakened the inhibition of adipogenic key genes and reduced the promotion of browning-related genes in bovine subcutaneous adipocytes. To sum up, our findings indicate that GH inhibits adipocyte differentiation and promotes adipocyte browning by regulating STAT5B in bovine subcutaneous adipocytes.

Introduction

As an important endocrine organ, adipose tissue can regulate energy balance and maintain metabolic homeostasis in the body [1]. Traditionally, there are two colors of adipose tissues in humans and animals, white and brown. Recent studies have found that the white adipocytes from white adipose tissue (WAT) can be converted into beige or brite adipocytes in response to external stimuli such as cold or drugs [2, 3]. Adipocytes with different colors have different functions, white adipocytes store energy, while brown and beige adipocytes consume energy to produce heat [4]. The main reason for the different metabolic types of adipocytes is the difference in mitochondrial content [5]. Compared with white adipocytes, brown and beige adipocytes have more mitochondria to produce heat through the uncoupling protein 1 (UCP1) rather than catalyzing ATP synthesis [6]. Since the process of browning can consume excess energy and reduce fat deposition, thus combating obesity and related metabolic diseases, more and more studies attempt to identify regulatory factors that can promote the browning of white adipocytes.

The differentiation of adipocytes is the key process for adipogenesis in adipose tissue and is regulated by many transcription factors. Peroxisome proliferator-activated receptor gamma (PPARγ) has been reported to significantly promote adipocyte differentiation, and the differentiation is inhibited by the knockout of PPARγ [7, 8]. Moreover, CCAAT enhancer binding protein alpha (CEBPα) is also a key transcription factor regulating adipogenic differentiation, which promotes differentiation combined with PPARγ [9]. In adipocytes cultured *in vitro*, the differentiation of adipocytes is often induced by a medium containing MDI (insulin + IBMX + dexamethasone). Notably, increasing evidence reveals that adding triiodothyronine, CL316243, and rosiglitazone to the medium during the induction stage of differentiation

can induce white adipocytes to become beige adipocytes [10–12]. This provides a new insight for the study on inducing the browning of adipocytes *in vitro*.

Growth hormone (GH) is a kind of polypeptide hormone, which participates in the regulation of body growth and development. GH possesses multiple biological functions, especially regulating fat deposition in adipose tissue. Previous studies have shown that GH treatment can significantly reduce the fat mass of mice [13, 14]. A study on human subcutaneous adipose tissue has also reported that GH supplementation markedly reduced the size of adipocytes [15]. These studies indicate that GH level is negatively correlated with adipose tissue content. In addition, research has disclosed that GH has a significant impact on fat distribution, and the content of subcutaneous WAT in GH knockout mice is quite higher than that in perigonadal WAT [16]. Previous evidence also identified that the extra-/intraperitoneal WAT ratio was upregulated in GH receptor antagonist (GHA) mice [17]. Although the inhibitory effect of GH on fat deposition has been widely confirmed, the effect of GH on adipocytes is still controversial. Accumulating evidences have reported that GH can promote the adipocyte differentiation of 3T3-L1 and 3T3-F442A, the cell lines from mice [18–20]. Inversely, the adipogenic differentiation of preadipocytes in primary cultures has been significantly inhibited during GH treatment [21, 22]. Furthermore, GH is also important in inducing WAT browning. For example, GH remarkably increases the expression level of UCP1 gene in subcutaneous WAT from mice [23]. To date, the ability of GH to induce the browning of adipocytes, especially subcutaneous adipocytes, has not been extensively studied.

Signal transducers and activator of transcription 5 (STAT5) protein plays a pivotal role in the regulation of fat deposition by GH. Study has shown that STAT5 is activated in GH-treated mouse adipose tissue [24]. After STAT5 knockout, GH could not continue to stimulate lipolysis in mouse adipocytes and the fat mass was also increased in mice [25, 26]. STAT5 is divided into two subtypes, STAT5A and STAT5B. STAT5A mainly acts on mammary tissue, and researchers have found that STAT5A-deficient mice could not lactate normally [27]. It has been reported that STAT5A plays a role in promoting adipogenesis [28]. In contrast, STAT5B has been reported to inhibit adipocyte differentiation [29]. Therefore, the regulation of GH on STAT5B may be the key for GH to inhibit the differentiation of preadipocytes in primary cultures.

Although the lipolysis of GH on adipose tissue has been established, the effect of GH on the differentiation and browning of bovine subcutaneous adipocytes remains unclear. In this study, we treated bovine subcutaneous preadipocytes with GH to detect the indicators related to differentiation and browning, and measured the expression of STAT5B protein during GH treatment. These results provide a theoretical basis for future studies into the mechanism of GH on fat metabolism of bovine subcutaneous adipocytes.

Materials And Methods

Materials and reagents

GH was purchased from Prospec (Ness-Ziona, Israeli). Phosphate buffer saline (PBS), penicillin and streptomycin, and Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12) were obtained

from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Sciencell (Carlsbad, CA, USA). Type I collagenase, insulin, rosiglitazone, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and oil red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). MitoTracker Red was purchased from Warbio (Nanjing, Jiangsu, China). Cell counting kit 8 (CCK-8), BCA assay kit, and ECL kit were purchased from Vazyme (Nanjing, Jiangsu, China). Bovine serum albumin (BSA), DAPI, RIPA buffer, and protease inhibitor were purchased from Beyotime (Shanghai, China). Genomic DNA Extraction Kit, Trizol, PrimeScript™ RT Master Mix, and SYBR Premix were purchased from TaKaRa (Tokyo, Japan). STAT5-IN-1 was purchased from Selleck (Houston, Texas, USA).

Cell culture and adipogenic differentiation

Bovine subcutaneous primary adipocytes were isolated from the subcutaneous adipose tissue in Simmental cattle. Firstly, the isolated adipose tissue samples were washed with PBS, minced with scissors, and digested with type I collagenase at 37 °C for 1 h. Next, the digested cell suspensions were filtered through the cell strainer, then centrifuged, re-suspended in a standard medium. The standard medium was DMEM/F12 supplemented with 10% FBS and 100 U/mL penicillin and streptomycin. Finally, the preadipocytes were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The culture medium was changed every 2 days. To induce adipogenic differentiation, the fully confluent preadipocytes were cultured in a standard medium for 2 days (day 0), then cultured in a differentiation medium for 6 days and finally cultured in a standard medium containing 1 μg/mL insulin for 2 days. The differentiation medium comprised of standard medium supplemented with 1 μg/mL insulin, 2 μM rosiglitazone, 1 μM dexamethasone, and 0.5 mM IBMX.

Cell viability assay

Bovine subcutaneous preadipocytes were seeded in a 96-well plate and incubated at 37℃ for 24 h. Next, the preadipocytes were treated with GH of various concentrations (0, 10, 100, 500 ng/mL) for 48 h. Afterward, 10 μL of CCK8 solution was added to each well and incubated at 37℃ for 4 h. The absorbance value was measured at 450 nm by using a microplate reader (Tecan, Switzerland).

Oil red O staining

After 8 days of adipogenic differentiation, adipocytes were washed with PBS and fixed in 4% paraformaldehyde for 30 min. Adipocytes were then washed with PBS three times and stained with oil red O working solution for 1 h at room temperature in dark. After washing with doubly distilled water 3-6 times, adipocytes were observed and photographed with a light microscope (CKX41, Olympus, Japan). Lipid contents in adipocytes were extracted with 100% isopropanol and the absorbance value at 510 nm was read by a microplate reader (Tecan, Switzerland).

Real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from adipocytes by using Trizol. After that, total RNA was used as a template for cDNA synthesis by using PrimeScript™ RT Master Mix. RT-qPCR was performed with the mixture of SYBR Premix, primers, and cDNA on a QuantStudio 5 system (ABI, Carlsbad, CA, USA). Details of the primer sequences for acetyl-CoA carboxylase (ACC), stearoyl-Coenzyme A desaturase 1 (SCD1), sterol regulatory element binding protein 1 (SREBP1), PPARγ, CEBPα, UCP1, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), PR domain containing 16 (PRDM16), cluster of differentiation 137 (CD137), T-box transcription factor 1 (TBX1), cell death inducing DFFA like effector A (CIDEA), deiodinase-2 (DIO2), mitochondrial transcription factors A (TFAM), nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The expression level of relative genes was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH [30].

Table 1

Primers sequences for target and reference genes.

Gene	Gene ID	Primer sequences (5' to 3')	Product size (bp)
ACC	NM_174224.2	F: GCTATGGAAGTCGGCTGTGGAAG	102
		R: GGAAGAGGCGGATGGGAATTGC	
SCD1	NM_173959.4	F: CTACACAACCACCACCACCATCAC	116
		R: CTCTCATTTCAGGGCGGATGTCTTC	
SREBP1	NM_001113302.1	F: CTCCGACACCACCAGCATCAAC	122
		R: GCAGCCCATTCATCAGCCAGAC	
PPARγ	NM_181024.2	F: TTGACCCAGAGAGTGAGCCCTTC	117
		R: CCACGGAGCTGATCCCAAAGTTG	
CEBPα	NM_176784. 2	F: TGGACAAGAACAGCAACGAGTACC	138
		R: GGCGGTCATTGTCACTGGTCAG	
UCP1	NM_001166528.1	F: TGCGTGGCTGACATAATCACCTTC	96
		R: GGCACTGGAGATCAGGCATTCG	
PGC-1α	XM_024993058.1	F: AGGCAGAGGCAGAAGGCAATTAAC	117
		R: CCTCAGTTCTGTCCGTGTTGTGTC	
PRDM16	XM_024976786.1	F: CTTGGAGCAGCACATGGTCGTC	101
		R: TCTGGTGGCGGATGAGGTTGG	
CD137	NM_001035336.2	F: TGGCGTCCTTCCTGGTTCTCC	108
		R: CCTCTTGGGCTGTTTGTACTGGTC	
TBX1	XM_024977887.1	F: GCAGTCACCGCCTATCAGAATCAC	84
		R: GTCACAGTCTCGGAAGCCTTTGG	
CIDEA	NM_001083449.1	F: CCTTCCGTGTCTCCAACCATGAC	100
		R: GCGACCACCAGTGCATCCAAG	
DIO2	NM_001010992.7	F: CATCCGTGGCTGACTTCCTGTTG	117
		R: CTTCCTGGTTCCGGTGCTTCTTC	
TFAM	NM_001034016.2	F: AAACCGAAAAGACCTCGCTCAGC	81
		R: TACCTGTGATGTGCCATCCCTAGC	
NRF1	NM_001098002.2	F: AATTATTCGGCGGTGGCTGATGG	82
		R: GCGTTGTCTGGATGGTCATCTCAC	
NRF2	NM_001011678.2	F: TCAGCCAGCACAACACATACCATC	128

		R: ACGGGAATGTCTCTGCCAAAAGC	
ND1	NC_006853.1	F: CGTAGAATATGCAGCAGGACCA	125
		R: GTTCTGGTATGTGTGGATTGTGG	
GAPDH	NM_001034034.2	F: CGGCACAGTCAAGGCAGAGAAC	116
		R: CCACATACTCAGCACCAGCATCAC	

ACC, acetyl-CoA carboxylase; SCD1, stearoyl-Coenzyme A desaturase 1; SREBP1, sterol regulatory element binding protein 1; PPARγ, peroxisome proliferator-activated receptor gamma; CEBPα, CCAAT enhancer binding protein alpha; UCP1, uncoupling protein 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PRDM16, PR domain containing 16; CD137, cluster of differentiation 137; TBX1, T-box transcription factor 1; CIDEA, cell death inducing DFFA like effector A; DIO2, deiodinase-2; TFAM, mitochondrial transcription factors A; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; ND1, NADH dehydrogenase subunit 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Western blot analysis

The lysates of adipocytes were harvested using the mixture of RIPA buffer and protease inhibitor. After centrifugation, the supernatant was quantified by a BCA assay kit and boiled with sodium dodecyl sulfate sample buffer. Then, the isolated protein was loaded and run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with fat-free milk followed by incubation with primary antibodies against PPARγ (SC-7196, Santa Cruz, 1:1000), GAPDH (10494-1-AP, Proteintech, 1:1000), STAT5B (bs-1142R, Bioss, 1:1000), and phosphor-STAT5B (bs-5703R, Bioss, 1:1000). The membranes were incubated with secondary antibody (7074P2, CST, 1:4000) and visualized using an ECL kit on the ChemiDocTM imaging system (BIO-RAD, Hercules, CA, USA).

Mitochondrial DNA (mtDNA) quantification

Adipocytes were treated with GH and performed for DNA extraction using the genomic DNA extraction kit. The mtDNA content was quantitatively analyzed by normalizing the NADH dehydrogenase subunit 1 (ND1) gene to GAPDH using RT-qPCR. The primer sequences are available in Table 1. The mtDNA content was calculated by using the $2^{-\Delta\Delta Ct}$ method [30].

MitoTracker red staining

Bovine subcutaneous adipocytes were differentiated into mature adipocytes in 6-well plates with coverslips on the bottom. Mature adipocytes were treated with 500 ng/mL GH for 6 h and stained with 200 nM MitoTracker red at 37°C for 30 min. After fixing with 4% paraformaldehyde and washing with PBS, adipocytes were stained with DAPI for 10 min. Finally, coverslips containing labeled adipocytes were

mounted on microscopic slides and the images were obtained with a laser scanning confocal microscope (LSM900, Zeiss, Germany).

Immunofluorescent staining

Adipocytes were differentiated in culture plates with coverslips and then treated with GH for 1 h. Next, adipocytes were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 30min. After blocking with 5% BSA for 1 h, adipocytes were incubated with STAT5B antibody (bs-1142R, Bioss, 1:200) overnight at 4 °C followed by staining with the florescent secondary antibody for 50 min. Then, the nuclei of adipocytes were counterstained with DAPI for 5 min. Fluorescence images were taken with laser scanning confocal microscope (LSM900, Zeiss, Germany).

Statistical analysis

All data were analyzed by the independent sample t-test using SPSS 22.0 software (IBM, Chicago, IL, USA). Data were shown as mean ± standard error of the mean (SEM). The *p*-values less than 0.05 were considered statistically significant.

Results

GH inhibited the differentiation of bovine subcutaneous preadipocytes

Firstly, we detected the cytotoxicity of GH on bovine subcutaneous preadipocytes by CCK8 assay. After GH treatment for 48 h, the cell viability of preadipocytes was unchanged at concentrations as high as 500 ng/mL (Fig. 1a). To investigate the effect on the differentiation of preadipocytes to mature adipocytes by GH treatment, we treated preadipocytes with different concentrations (0, 10, 100, and 500 ng/mL) of GH for 8 days during adipocyte differentiation (Fig. 1b). Oil red O staining showed clear lipid droplets in differentiated adipocytes without GH treatment (Fig. 1c). However, treatment with GH for 8 days inhibited the accumulation of lipid droplets in differentiated adipocytes (Fig. 1c). Lipid extraction of adipocytes after staining with oil red O showed that 100 and 500 ng/mL GH treatments significantly reduced the lipid content of differentiated adipocytes (Fig. 1d). To further demonstrate the inhibitory effect of GH on adipogenesis, we examined the expression levels of adipogenesis-related genes (ACC, SCD1, SREBP1, PPARγ, and CEBPα) in differentiated adipocytes after 8 days of GH treatment. Compared with those in the 0 ng/mL GH treatment group, 10 ng/mL GH treatment significantly inhibited the expression level of SREBP1 gene, 100 ng/mL GH treatment significantly decreased the expression level of PPARγ gene, and the expression levels of SCD1, SREBP1, PPARγ, and CEBPα genes were significantly decreased in 500 ng/mL GH treatment group (Fig. 1e). These results suggested that GH inhibited the differentiation of bovine subcutaneous preadipocytes.

GH strongly suppressed the differentiation of bovine subcutaneous adipocytes at the early stage of differentiation

To further understand the inhibitory mechanism of GH on the differentiation of adipocytes, we treated adipocytes with GH at different stages of adipogenic differentiation (Fig. 2a). Adipogenesis of adipocytes was detected by oil red O staining. Compared with untreated adipocytes, the lipid content was significantly decreased in adipocytes treated with 500 ng/mL GH at different stages (Fig. 2b-c). Interestingly, we observed that the inhibitory effect of GH on the early stage of adipocyte differentiation (0-2 days) was stronger compared to the later stage of adipocyte differentiation (2-8 days). Therefore, adipocytes were collected for analysis of adipogenic key genes and proteins after treatment with differentiation medium and GH for 1 and 2 days. RT-qPCR results showed that the expression levels of SCD1, SREBP1, PPARγ, and CEBPα were significantly reduced after GH treatment for 1 day, and the expression levels of ACC, SCD1, SREBP1, PPARγ, and CEBPα were significantly decreased after GH treatment for 2 days (Fig. 2d-e). Western blot analysis showed that the expression level of PPARγ protein decreased significantly after GH treatment for both 1 and 2 days (Fig. 2f).

GH promoted the browning and mitochondrial biogenesis of bovine subcutaneous adipocytes

To study the effect of GH on the browning of mature adipocytes, we cultured adipocytes in a differentiation medium for 8 days and then treated the differentiated adipocytes with GH. The expression level of the browning key gene UCP1 was analyzed by RT-qPCR. As shown in Fig. 3a, the expression level of UCP1 gene was significantly increased in fully differentiated adipocytes treated with 500 ng/mL GH for 2 h and 6 h. Then, we examined the expression levels of browning-related genes (PGC-1α, PRDM16, CD137, TBX1, CIDEA, and DIO2) in mature adipocytes treated with 500 ng/mL GH for 6 h, and found that GH treatment significantly increased the expression levels of PGC-1α, PRDM16, TBX1, and CIDEA genes (Fig. 3b). Mitochondrial biogenesis is one of the key characteristics of browning. We observed that 6 h of GH treatment significantly increased the content of mtDNA in mature adipocytes, suggesting the increased mitochondrial number (Fig. 3c). MitoTracker red staining in mature adipocytes further demonstrated that GH treatment enhanced mitochondrial red fluorescence signal (Fig. 3d). In addition, mitochondrial biogenesis-specific markers NRF1 and NRF2 were up-regulated in GH-treated mature adipocytes (Fig. 3e). Together, the above results showed that GH promoted the browning and mitochondrial biogenesis of bovine subcutaneous adipocytes.

GH increased the phosphorylation of STAT5B in bovine subcutaneous adipocytes

STAT5 is widely involved in growth and development processes including cell proliferation, differentiation, etc. As a subtype of STAT5, STAT5B can participate in the GH-regulated lipolysis process [31, 32]. Therefore, to clarify whether GH can activate the expression of STAT5B in bovine subcutaneous adipocytes, we first treated preadipocytes (day 0) with a differentiation medium and 500 ng/mL GH for indicated time to detect the phosphorylation level of STAT5B. Although incubation with GH for 10 min and 30 min had no significant effect on the phosphorylation level of STAT5B, 60 min and 180 min of GH treatment significantly increased the phosphorylation level of STAT5B (Fig. 4a). Then we treated fully differentiated adipocytes with 500 ng/mL GH for 1 h and 6 h and found that both 1 h and 6 h of GH treatment markedly promoted STAT5B activation (Fig. 4b). Furthermore, immunofluorescence staining of

STAT5B showed that STAT5B protein could be translocated to the nucleus to regulate gene transcription (Fig. 4c).

GH-induced differentiation and browning of bovine subcutaneous adipocytes were mediated by STAT5B

To further determine the activation mechanism of GH on STAT5B, we treated adipocytes in the differentiation stage (day 0) with GH for 1 h, with or without pretreatment with 100 μM STAT5-IN-1 (STAT5 inhibitor) for 1 h. Western blot analysis showed that 100 μM STAT5-IN-1 treatment significantly inhibited the phosphorylation of STAT5B initiated by GH (Fig. 5a). Moreover, we treated adipocytes with 100 μM STAT5-IN-1 for 1 h followed by GH for 24 h during the differentiated process (day 0). We found that pretreatment with STAT5-IN-1 significantly alleviated the downregulation of PPARγ and SREBP1 genes after GH treatment (Fig. 5b). In addition, treatment on fully differentiated adipocytes with STAT5-IN-1 and GH for 1 h and 6 h, respectively, significantly inhibited GH-induced the upregulation of UCP1 and PGC-1α genes. These results indicated that GH may inhibit adipogenic differentiation and promote the browning of adipocytes by activating STAT5B.

Discussion

GH is an important cytokine in regulating the growth and development of the body, and accumulating evidences have shown that GH has a significant impact on adipose tissue metabolism [33, 34]. In particular, GH can suppress the differentiation of primary cell cultures [35]. Indeed, although GH has been found to promote the expression of UCP1 in mouse model, the specific regulation mechanisms need further research [23]. STAT5B is a key transcription factor in cell development regulated by GH, which can transmit extracellular signals to the nucleus and regulate cell metabolic activities [36]. Therefore, in this research, we investigated the effects of GH on the differentiation and browning of bovine subcutaneous adipocytes by culturing bovine subcutaneous adipocytes *in vitro*, and explored the role of STAT5B protein in the regulation of GH on bovine subcutaneous adipocytes.

It has been well accepted that GH can promote lipolysis and reduce WAT weight in the body [37]. Research on primary human mesenchymal stromal cells (MSCs) has confirmed that GH inhibited adipocyte differentiation [21]. In the present study, we found that GH treatment for 8 days significantly inhibited the lipid content of bovine subcutaneous adipocytes, which is consistent with the results of Zhao et al. [38]. Olarescu et al. [35] also found that bovine GH (bGH) cells from mice subcutaneous WAT displayed a decreased lipid accumulation compared to wild-type (WT) cells. Adipocyte differentiation is promoted by many key transcription factors, among which PPARγ is the core regulator of entire terminal differentiation [39]. In addition, CEBPα promotes adipogenic differentiation through reciprocal positive feedback regulation with PPARγ [39]. SREBP1 can promote the expression of PPARγ and SCD1, and both SREBP1 and SCD1 play an important role in regulating de novo lipogenesis [40, 41]. Our results suggested that GH inhibited adipogenesis of bovine subcutaneous adipocytes by down-regulating the expression levels of PPARγ, CEBPα, SREBP1, and SCD1 genes. This is contrary to the results in 3T3-L1 adipocytes, in which GH can significantly elevate the expression level of PPARγ protein [18, 42]. This may

further confirm the opposite effects of GH on cell lines and primary adipocytes. The early phase of differentiation is the most crucial phase to initiate and ensure the normal differentiation process. Our study revealed that GH treatment had a stronger inhibitory effect on lipid accumulation in the early stage (0–2 days) than that in the later stage (2–8 days). Additionally, we identified the inhibitory effect of GH on the expression of key genes and proteins during adipogenic differentiation. These findings suggested that GH exerted the ability to inhibit adipogenic differentiation, especially in the early stage of differentiation, through inhibiting lipid accumulation and the expression of adipogenic-related genes.

The browning of WAT is essential for the body to combat obesity, resist cold and increase heat production. Accordingly, researches related to inducing browning by using *in vitro* and *in vivo* models have attracted increasing attention. Recent work showed that compared with WT mice, the expression of UCP1 protein in bGH transgenic mice was increased [43]. As a marker of beige adipocytes, UCP1 leads protons to leak through the inner membrane of mitochondria and thus converting electrochemical energy into heat, which plays a central role in heat production [44]. Our study demonstrated that GH treatment could significantly increase the expression levels of UCP1 and browning-related key genes (PGC-1α, PRDM16, TBX1, and CIDEA) in bovine subcutaneous adipocytes, indicating a potential role of GH in inducing beige fat-like characteristic. Moreover, Hayashi et al. [45] also proposed that GH induced the expression of UCP1 protein in 3T3-L1 cells, which was coincident with our results. Due to beige adipocytes requiring more mitochondria for metabolism and thermogenesis, mitochondrial biogenesis has become a marker process of browning. Research has shown that mitochondrial biogenesis is dominant in the process of beige adipocytes formation [46]. The mtDNA replication is the main feature of mitochondrial biogenesis, which reflects the increase of mitochondrial number. In this study, GH treatment can increase mtDNA contents in bovine subcutaneous adipocytes. In addition, MitoTracker staining further demonstrated that GH treatment promoted mitochondrial biogenesis in bovine subcutaneous adipocytes. TFAM, NRF1, and NRF2 are key regulatory factors controlling mitochondrial biogenesis, and PGC-1α can activate the expression of these key transcription factors, led to an increase in mtDNA content, thus promoting mitochondrial biogenesis [47, 48]. In this work, we demonstrated that the expression levels of PGC-1α, NRF1, and NRF2 genes were increased in GH-treated adipocytes. Overall, these findings indicated that GH can induce the browning of bovine subcutaneous adipocytes and promote the mitochondrial biogenesis.

There is increasing evidence that GH has the potential to activate STAT5B both *in vivo* and *in vitro* [18, 49]. Previous data have suggested that the expression of STAT5B protein was increased during adipocyte differentiation, and STAT5B knockdown up-regulated the expression of PPARγ and CEBPα [29, 50]. Moreover, several studies also confirmed that STAT5 promoted transcription by binding to the UCP1 promoter and cold exposure could not promote UCP1 protein increase in STAT5-deficient mice [45, 51]. Therefore, we hypothesized that GH could activate STAT5B expression to regulate the differentiation and browning of bovine subcutaneous adipocytes. As expected, our results showed that GH can activate STAT5B in differentiating as well as full differentiated adipocytes, suggesting that STAT5B plays an important role in GH-regulated differentiation and browning processes. STAT5B is widely present in the cytoplasm as a monomer when cells are not stimulated by cytokines and hormones. In response to GH

stimulation, phosphorylated STAT5B separates from its receptor and forms different polymeric forms, which are transferred to the nucleus and activate or inhibit transcription by binding to specific DNA sequences [52]. This transcriptional regulation of nuclear translocation is the key for STAT5B to fulfill its function. In this study, STAT5B protein was translocated to the nucleus after GH treatment in bovine subcutaneous adipocytes. Consistent with our findings, it has been reported that GH promotes mitochondrial biogenesis by activating STAT5, which is then transported to the nucleus and interacts synergistically with other growth factors [53]. Next, we used STAT5-IN-1 to block the activity of STAT5B and found that the expression level of key genes involved in adipocyte differentiation and browning decreased significantly, indicating that GH inhibits adipocyte differentiation and promotes adipocyte browning *in vitro* by activating the expression of STAT5B protein.

Conclusion

In conclusion, our data prove that GH can inhibit the adipogenic differentiation of bovine subcutaneous preadipocytes, and the inhibitory effect of GH is more obvious in the early stage of differentiation than that in the later stage. GH can also promote the browning of bovine subcutaneous adipocytes, accompanied by mitochondrial biogenesis. In addition, studies on STAT5B, a key factor in transcriptional regulation, found that GH regulated the differentiation and browning process of bovine subcutaneous adipocytes through activating STAT5B. Collectively, our results suggest the inhibitory effect of GH on the differentiation of preadipocytes and the feasibility of GH to induce the browning of adipocytes *in vitro*.

Declarations

Author contributions

TL performed the main experiments and drafted the manuscript. TL and HB designed this study. TL and HF carried out the data collection and analysis. TL and LY performed the material preparation. PY supervised the research and revised the manuscript. All authors reviewed and approved the final manuscript.

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Data availability

The data that support the findings of the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The collection of adipose tissue samples were approved by the Institutional Animal Care and Use Committee at Nanjing Agricultural University (Permission number: SYXK-2017-0027).

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Figures

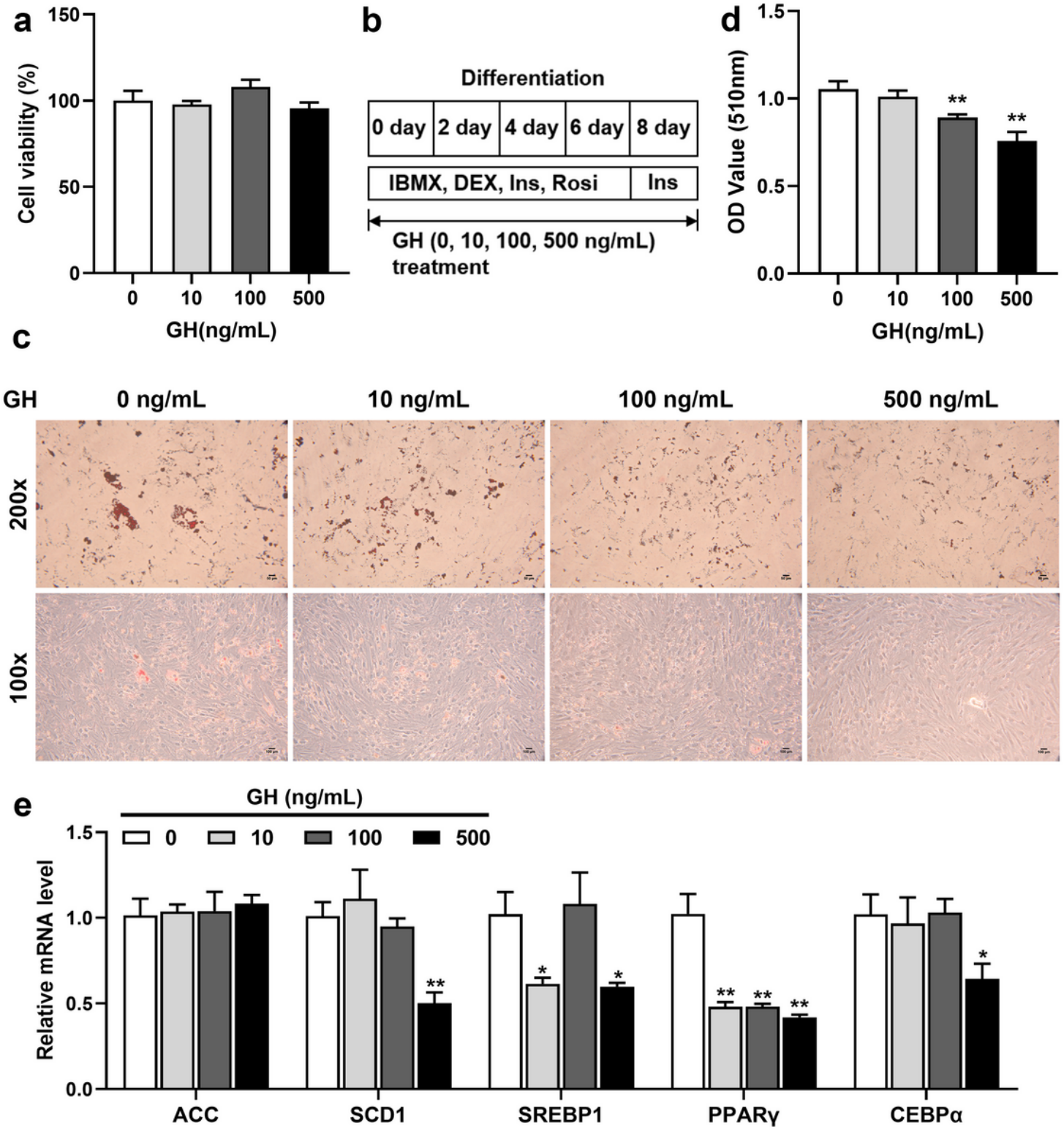

Figure 1

GH inhibited the differentiation of bovine subcutaneous preadipocytes. **a** Cell viability of bovine subcutaneous preadipocytes treated with different concentrations (10, 100, 500 ng/mL) of GH for 48 h. **b** GH treatment (10, 100, 500 ng/mL) during the adipogenic differentiation program. **c** Oil red O staining of bovine subcutaneous adipocytes on day 8 after treatment with various concentrations of GH. Magnification, 200x and 100x. Scale bars, 50 μm and 100 μm. **d** The result of oil red O extraction. **e** The

expression levels of adipogenic key genes was detected by RT-qPCR in adipocytes after GH treatment for 8 days. Data are presented as means ± SEM (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the 0 ng/mL group.

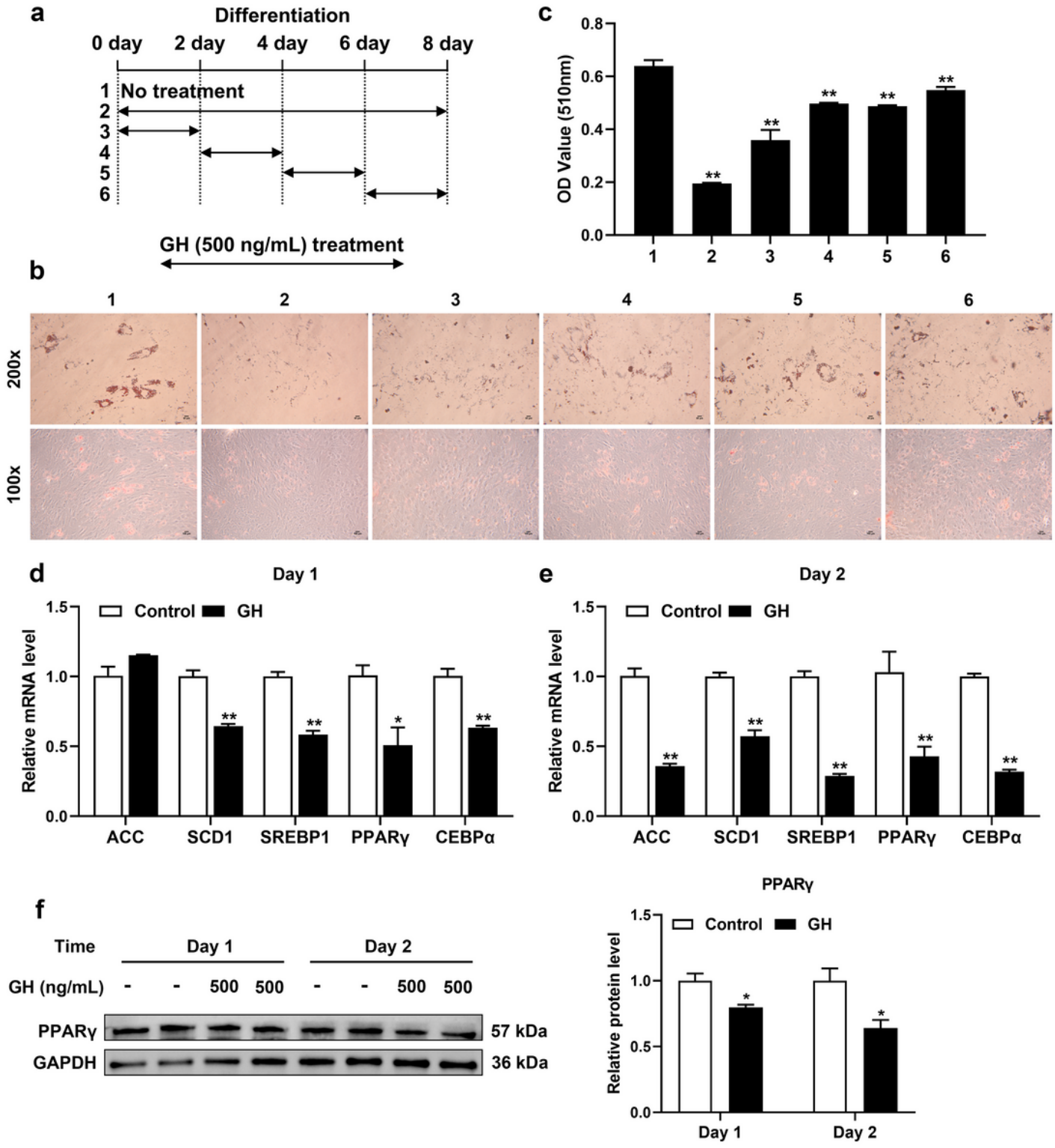

Figure 2

GH strongly suppressed the differentiation of bovine subcutaneous adipocytes at the early stage of differentiation. **a** Adipocytes were treated with GH (500 ng/mL) at the indicated time point during the

differentiated process. **b** Oil red O staining of differentiated bovine subcutaneous adipocytes. Magnification, 200x and 100x. Scale bars, 50 μm and 100 μm. **c** The content of oil red O extraction. **d, e** GH-treated adipocytes were collected for RT-qPCR analysis at 1 and 2 days of differentiation. **f** Western blot analysis of PPARγ at 1 and 2 days of differentiation. Data are presented as means ± SEM (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group.

Figure 3

GH promoted the browning and mitochondrial biogenesis of bovine subcutaneous adipocytes. **a** The expression level of UCP1 gene in full differentiated adipocytes treated with 500 ng/mL GH for the indicated times. **b** After 6 h of GH treatment, the expression of browning-related genes was analyzed by RT-qPCR. **c** Mitochondrial DNA content quantification by RT-qPCR. **d** Mature bovine subcutaneous adipocytes were stained with MitoTracker red. Magnification, 200x. Scale bars, 50 μm. **e** The mRNA expression of TFAM, NRF1, and NRF2 genes in mature bovine subcutaneous adipocytes treated with 500 ng/mL GH for 6 h. Data are presented as means ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group.

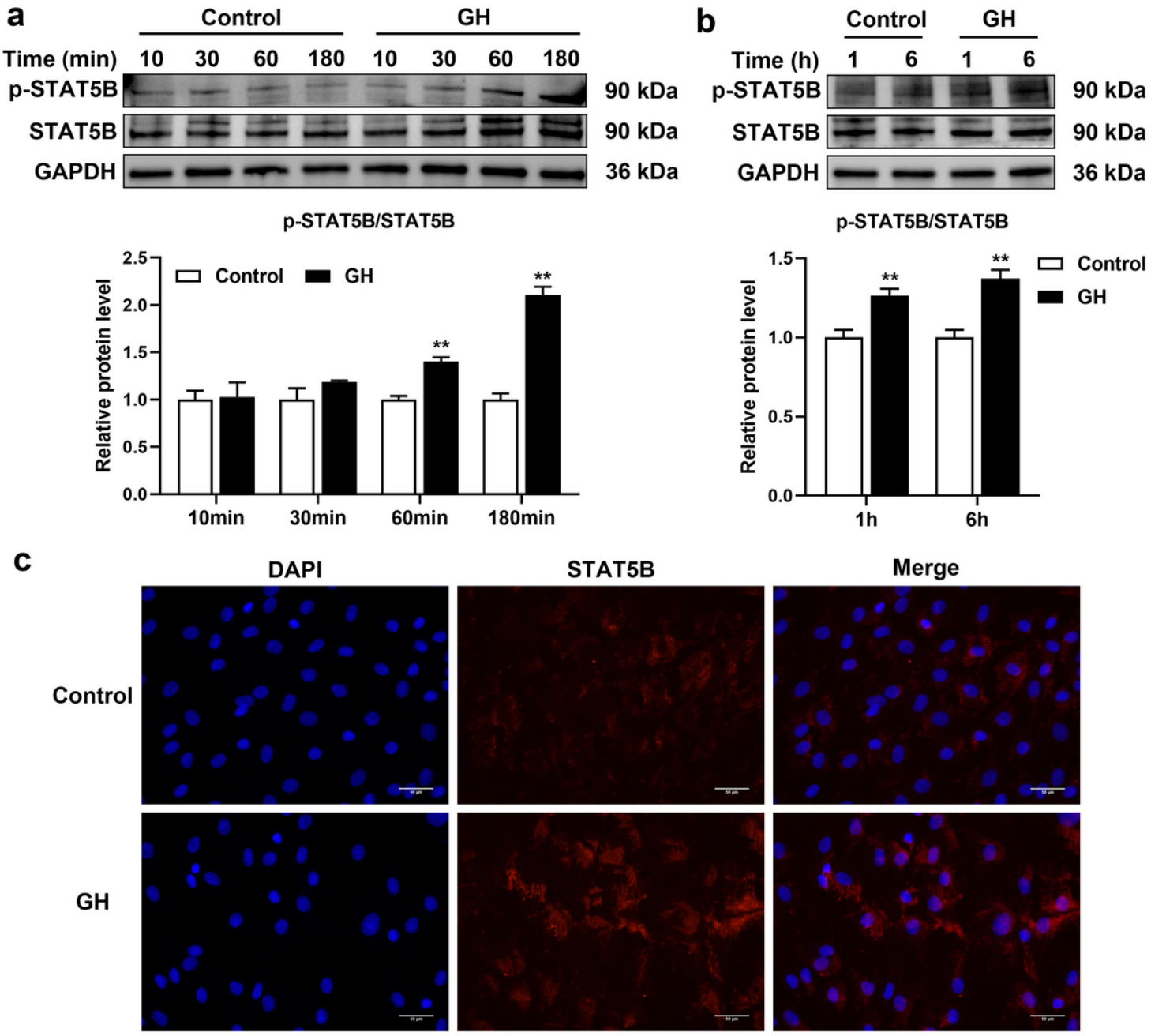

Figure 4

GH increased the phosphorylation of STAT5B in bovine subcutaneous adipocytes. **a** Western blot analysis of p-STAT5B and STAT5B at day 0 of adipocyte differentiation with GH (500 ng/mL) treatment for 10, 30, 60, and 180 minutes. **b** Western blot analysis of p-STAT5B and STAT5B in mature adipocytes after GH (500 ng/mL) treatment for 1 and 6 hours. **c** Images of adipocytes STAT5B immunofluorescent staining after GH (500 ng/mL) treatment for 60 min. Magnification, 200x. Scale bars, 50 μm. Data are presented as means ± SEM (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group.

Figure 5

GH-induced differentiation and browning of bovine subcutaneous adipocytes were mediated by STAT5B. **a** Adipocytes were preincubated with STAT5-IN-1 (100 μM) for 1 h and treated with GH (500 ng/mL) for 1 h during the differentiated process (day 0). Western blot was used to analyze the expression of p-STAT5B and STAT5B proteins (n = 4). **b** Adipocytes were preincubated with STAT5-IN-1 (100 μM) for 1 h and treated with GH (500 ng/mL) for 24 h during the differentiated process (day 0). RT-qPCR was used to analyze the expression of PPARγ, CEBPα, and SREBP1 genes (n = 3). **c** Mature adipocytes were preincubated with STAT5-IN-1 (100μM) for 1 h and treated with GH (500 ng/mL) for 6 h. RT-qPCR was used to analyze the expression levels of UCP1, PGC-1α, and PRDM16 genes (n = 3). Data are presented as means ± SEM. Statistical significance shows as $^{*}P < 0.05$, $^{**}P < 0.01$.