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Thermogenesis and cellular senescence of diabetic adipocytes in response to β-agonists and 18-carbon fatty acids

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ABSTRACT

Purpose: Adipocyte dysfunction has been reported in diabetes, and stimulating thermogenesis and suppressing senescence in adipocytes potentially alleviates metabolic dysregulation. This study aimed to investigate thermogenesis and cellular senescence in diabetic adipocytes under basal conditions and in response to stimuli.
Methods: White and brown primary adipocytes derived from control (CON) and *db/db* (DB) mice were treated with β-agonists, such as norepinephrine (NE) and CL316,243, and 18-carbon fatty acids, including stearic acid, oleic acid (OLA), linoleic acid (LNA), and α-linolenic acid, and the expression of the genes related to thermogenesis and cellular senescence was measured.
Results: Although no difference in the thermogenic and cellular senescence gene expression in white adipose tissue (WAT) was noted between the CON and DB mice, brown adipose tissue (BAT) from the DB mice exhibited lower uncoupling protein 1 (*Ucp1*) expression and higher cyclin-dependent kinase inhibitor (*Cdkn)1a* and *Cdkn2a* expression levels compared to that from the CON mice. Stromal vascular cells isolated from the BAT of the DB mice displayed higher peroxisome proliferator-activated receptor gamma (*Pparg*), CCAAT/enhancer-binding protein alpha (*Cebpa*), *Cdkn1a*, and *Cdkn2a* expression levels. White adipocytes from the DB mice exhibited lower *Ucp1*, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*Pgc1a*), and PR domain containing 16 (*Prdm16*) expression levels regardless of β-agonist treatment. NE upregulated *Pgc1a* in both white and brown adipocytes from the CON mice, but not in those from the DB mice. Although none of the fatty acids were observed to downregulate the cellular senescence genes in fully differentiated adipocytes, the OLA-treated brown adipocytes derived from DB mice exhibited lower *Cdkn1a* and *Cdkn2b* expression levels than the LNA-treated cells.
Conclusion: These results indicate that the lower thermogenic capacity of diabetic adipocytes may be related to their cellular senescence, and different fatty acids potentially exert divergent effects on the expression of cellular senescence genes.

Keywords: diabetes mellitus; fatty acids; adrenergic beta-agonists; adipocytes; adipose-derived mesenchymal stromal cells

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Conflict of Interest
There are no financial or other issues that might lead to conflict of interest.

Author Contributions
Conceptualization: Shin S; Formal analysis: Park S, Ock SA, Park YJ, Shin S; Funding acquisition: Han SN, Shin S; Investigation: Park S, Ock SA, Park YJ, Han SN, Shin S; Supervision: Shin S; Writing – original draft: Shin S; Writing – review & editing: Shin S.

INTRODUCTION

Diabetes, one of the most prevalent metabolic disorders worldwide, is characterized by hyperglycemia induced by impaired insulin signaling or secretion [1]. Since thermogenic adipocytes, including brown and beige adipocytes, absorb glucose and fat from the blood circulation, activating thermogenic adipocytes reportedly enhances insulin signaling and improves glucose and lipid homeostasis [2,3]. However, in diabetes, hyperglycemia induces senescent cell accumulation in multiple organs, such as adipose tissues and the pancreas [4,5]. Senescent cells, characterized by irreversible cell cycle arrest caused by various cell stresses, secrete pro-inflammatory cytokines and chemokines, thus exerting a negative effect on organ function and leading to metabolic changes [6,7].

Compared with young mice, naturally aged mice have been shown to exhibit lower expression levels of thermogenic genes, such as *Ucp1*, *Pgc1a*, and *Dio2*, and higher expression levels of cellular senescence genes, such as *Cdkn1a* (also known as *p21*), *Cdkn2a* (also known as *p16*), and senescence-associated β-galactosidase (SA-β-gal) [8]. When *Cdkn1a* overexpression was induced in adipose progenitor cells (APCs) or uncoupling protein 1-positive (UCP1+) cells, the mice developed impaired beige fat formation and worse glycemia under cold exposure or β-agonist treatment [8,9]. In contrast, *Cdkn2a* deficiency increased energy expenditure by promoting adipose thermogenesis and improved insulin sensitivity in high-fat diet (HFD)-fed mice [10]. In the APCs of old mice, *Cdkn2a* deletion restored cold-induced beige fat formation [8], and that in UCP1+ cells induced the formation and expansion of beige adipocytes, even under room temperature or high-fat and high-sucrose diet feeding [9,11].

Similarly, injection of PD0332991 (an inducer of cellular senescence) suppressed beige adipocyte formation in young mice, whereas treatment with SB202190 (an inhibitor of cellular senescence) induced beige adipogenesis and reduced SA-β-gal expression in old human APCs [8]. These data indicate that an anti-senescence strategy could be applied to patients with diabetes [12]. In fact, metformin, an anti-diabetic agent that has been used for decades, reportedly suppresses stem cell senescence and extends life span in mice and *Caenorhabditis elegans* [13].

In contrast, the activation of β-adrenergic receptors (β-ARs) or peroxisome proliferator-activated receptors (PPARs) is required to induce thermogenesis in brown and beige adipocytes [14-16]. Treatment with β-agonists, such as norepinephrine (NE; a non-selective β-AR agonist) and CL316,243 (CL; a specific β_3-AR agonist), recruits transcription factors, including PR domain containing 16 (PRDM16) and PPARγ coactivator-1α, and upregulates thermogenic genes such as *Ucp1* and *Pgc1a* [14]. As PPAR agonists, long-chain fatty acids (LCFAs), especially n-3 polyunsaturated fatty acids, induce adipose thermogenesis by promoting brown and beige adipogenesis [15,16]. We previously reported the thermogenic effects of 18-carbon fatty acids, the most common LCFAs found in the human diet, in both *in vitro* and *in vivo* models [17-19].

Although β-agonists and 18-carbon fatty acids have been shown to activate thermogenesis in adipocytes and improve glucose and lipid homeostasis, their effects on diabetic adipocytes in terms of thermogenesis and cellular senescence are yet to be elucidated. Therefore, this study aimed to demonstrate the effects of diabetes on adipose thermogenesis and cellular senescence under basal conditions or in response to β-agonists and 18-carbon fatty acids.

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METHODS

Animals and diets

Six-week-old male C57BLKS/J-m^{+}/m^{+} (control; CON) and C57BLKS/J-*db*/*db* (DB) mice purchased from Japan SLC (Shizuoka, Japan) were fed a 10% kcal fat diet (#D12450B; Research Diets, New Brunswick, NJ, USA) *ad libitum* for 7 weeks under controlled temperature, humidity, and a light-dark cycle. Blood glucose levels were measured with a G-CARE glucometer (GC Medical Science, Yongin, Korea) using whole blood drawn from the mouse tail vein. At the end of the experimental period, the mice were euthanized by CO_2 asphyxiation after 12 hours fasting, and inguinal subcutaneous white adipose tissue (WAT) and brown adipose tissue (BAT) were dissected. All animal procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Seoul National University (No. SNU-201111-1-3).

Stromal vascular cell (SVC) isolation and differentiation

SVC, containing APCs, were isolated from pooled WAT and BAT of the mice as previously described [20], and all *in vitro* experiments were conducted with 3 technical replicates. A part of SVC was utilized to study characteristics of APCs, and the other part was fully differentiated into mature adipocytes. To induce adipogenic differentiation, the cells were grown in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Corning, Corning, NY, USA) and 1% penicillin and streptomycin (Sigma-Aldrich) at 37°C and 5% CO_2. Two days after confluence, cell differentiation was initiated with DMEM/F12 with 10% FBS, 1.7 μM insulin, 1 μM dexamethasone, 500 μM isobutylmethylxanthine, 1 μM rosiglitazone, 17 μM pantothenic acid, and 33 μM biotin for 6 days.

β-agonist treatment

To induce thermogenesis by stimulating β-adrenergic signaling, 10 μM of NE (Cayman, Ann Arbor, MI, USA) or CL (Cayman) was treated for 4 hours on fully differentiated adipocytes.

Fatty acid preparation and treatment

As described previously [17], 18-carbon fatty acids, including stearic acid (STA; 18:0; Cayman), oleic acid (OLA; 18:1n-9; Cayman), linoleic acid (LNA; 18:2n-6; Cayman), and α-linolenic acid (ALA; 18:3n-3; Cayman), were completely dissolved in 0.9% NaCl solution. Each solution was mixed with 20% bovine serum albumin (BSA; Sigma-Aldrich) in 0.9% NaCl to make the final concentration of the fatty acids to 5 mM and sterilized through a 0.2 μm syringe filter. To induce thermogenesis by stimulating PPAR, 50 μM of the fatty acids bound to BSA were treated for 24 hours on fully differentiated adipocytes.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1 μg of total RNA using the PrimeScript II 1st strand cDNA synthesis kit (Takara, Japan). Using TB Green Premix Ex Taq (Takara) and StepOnePlus Real-time PCR System (Applied Biosystems, Waltham, MA, USA), the mRNA levels of adipogenic genes (*Pparg* and *Cebpa*), thermogenic genes (*Ucp1*, *Pgc1a*, and *Prdm16*), and cell senescence genes (*Cdkn1a*, *Cdkn2a*, and *Cdkn2b*) were quantified and normalized relative to *18S* rRNA. Fold changes of gene expression were calculated by the ΔΔCt method. Specific primer sequences used are shown in **Table 1**.

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Table 1. Primer sequences used for quantitative real-time polymerase chain reaction

Genes	Function	Forward primer	Reverse primer
18S	Housekeeping gene	ATC CCT GAG AAG TTC CAG CA	CCT CTT GGT GAG GTC GAT GT
Pparg	Adipogenesis	TTG ACC CAG AGC ATG GTG C	GAA GTT GGT GGG CCA GAA TG
Cebpa	Adipogenesis	AAT GGC AGT GTG CAC GTC TA	CCC CAG CCG TTA GTG AAG AG
Cdkn1a	Cellular senescence	GAG AGG CCA CCA TTT GAG GA	GGT CCC ACG CCT ATG GAA TG
Cdkn2a	Cellular senescence	CAT CTG GAG CAG CAT GGA GTC	GTT GCC CAT CAT CAT CAC CTG AAT
Cdkn2b	Cellular senescence	GGC GCC CAA TCC AGG TCA T	CGT GCA CAG GTC TGG TAA
Ucp1	Thermogenesis	GGG CCC TTG TAA ACA ACA AA	GTC GGT CCT TCC TTG GTG TA
Pgc1a	Thermogenesis	GTC CTT CCT CCA TGC CTG AC	GTG TGG TTT GCT GCA TGG TT
Prdm16	Thermogenesis	GGC TCA AGG AGG AGG AGA GA	AGG TCC GGG TCA GGT TCA TA

18S, 18S ribosomal RNA; *Pparg*, peroxisome proliferator-activated receptor gamma; *Cebpa*, CCAAT enhancer binding protein alpha; *Cdkn1a*, cyclin dependent kinase inhibitor 1A (also known as p21); *Cdkn2a*, cyclin dependent kinase inhibitor 2A (also known as p16 or Ink4a); *Cdkn2b*, cyclin dependent kinase inhibitor 2B (also known as p15 or Ink4b); *Ucp1*, uncoupling protein 1; *Pgc1a*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Prdm16*, PR domain containing 16.

Statistical analysis

Student's t-test or two-way analysis of variance with Tukey's post hoc test was performed to determine significant differences among groups. Pearson's correlation was used to determine the linear relationship between variables. Differences were considered statistically significant at $p < 0.05$. Data were presented as means ± SEM, and analyzed using SPSS version 24 (SPSS Inc., Armonk, NY, USA).

RESULTS

Body weight, food intake, blood glucose, and tissue weight

Compared with CON mice, DB mice consumed more calories and gained more weight during the experimental period. DB mice also exhibited greater body weight, higher blood glucose levels, and more abundant WAT and BAT than CON mice at the end point (**Table 2**).

Thermogenesis and cellular senescence in adipose tissues

To ascertain whether diabetes alters thermogenesis and cellular senescence in whole adipose tissues, the mRNA expression levels of related genes were measured in WAT and BAT (**Fig. 1**). Although no significant difference in thermogenic and cellular senescence gene expression in WAT was observed between the two groups, DB mice displayed significantly lower *Ucp1* and higher *Cdkn1a* and *Cdkn2a* expression levels in WAT and tended to exhibit lower *Pgc1a* and *Prdm16* expression levels in BAT than CON mice.

Table 2. Body weight, food intake, blood glucose, and tissue weight of the mice fed experimental diets

Variables	CON	DB	p-value
Body weight at 6 wk (g)	17.66 ± 0.47	25.96 ± 0.80*	< 0.001
Body weight at 13 wk (g)	22.68 ± 0.45	36.43 ± 1.11*	< 0.001
Body weight gain (g)	5.02 ± 0.34	10.48 ± 1.09*	< 0.001
Food intake (g/day)	2.71 ± 0.06	5.15 ± 0.53*	0.003
Food intake (kcal/day)	10.41 ± 0.23	19.81 ± 2.03*	0.003
Blood glucose at 6 wk (mg/dL)	173.29 ± 11.72	197.86 ± 17.37*	< 0.001
Blood glucose at 13 wk (mg/dL)	379.14 ± 59.62	590.57 ± 25.78*	< 0.001
White adipose tissue[1)] (g/100g body weight)	1.79 ± 0.23	10.47 ± 0.26*	< 0.001
Brown adipose tissue (g/100g body weight)	0.51 ± 0.04	0.82 ± 0.06*	0.001

Data are presented as means ± SEM (n = 7 for each group).
CON, control; DB, *db/db*.
*Indicate significant differences at $p < 0.05$.
[1)]Inguinal subcutaneous white adipose tissue.

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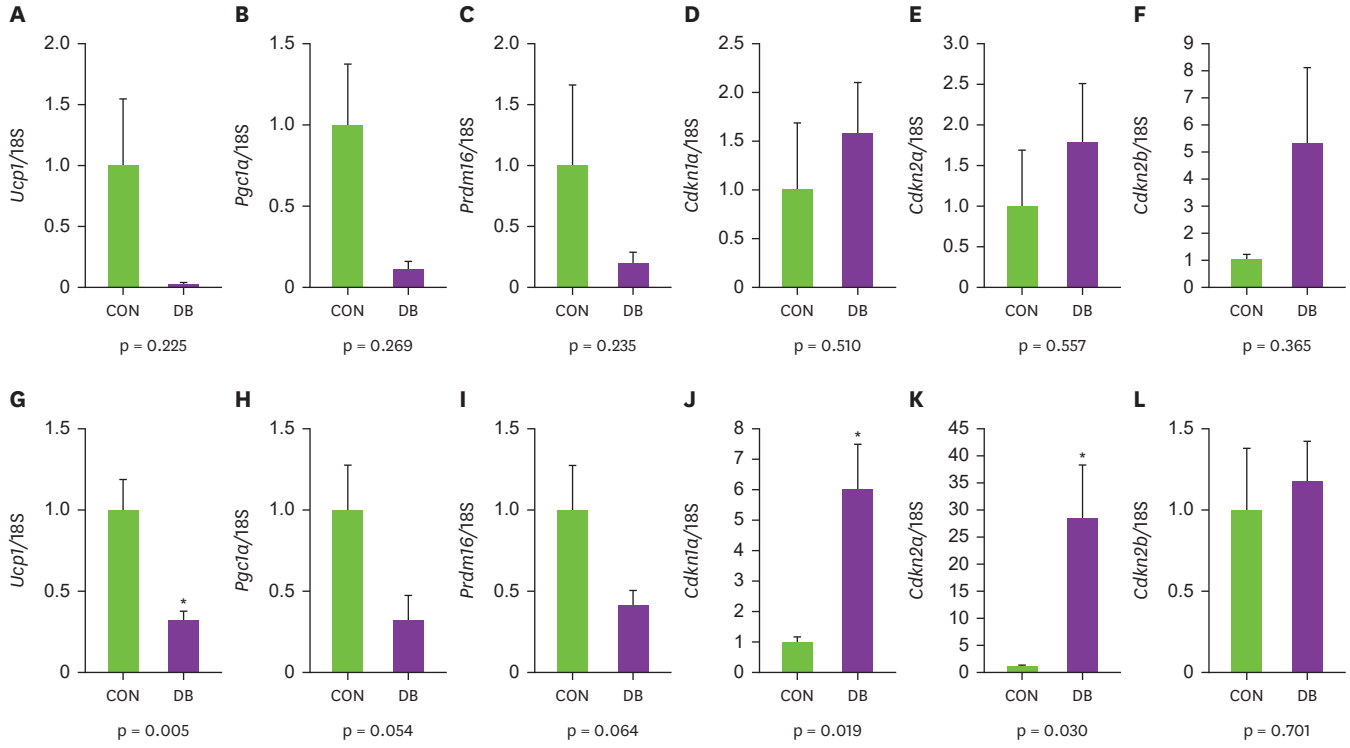

Fig. 1. Effects of diabetes on thermogenic and senescence gene expression in adipose tissues.
(A-C) Thermogenic genes in WAT, (D-F) senescence genes in WAT, (G-I) thermogenic genes in BAT, and (J-L) senescence genes in BAT. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 7, biological replicates).
CON, control; DB, *db/db*; WAT, white adipose tissue; BAT, brown adipose tissue.
*Indicate significant differences at $p < 0.05$ by Student's t-test.

The expression levels of *Ucp1* and *Pgc1a* in BAT were negatively correlated with body weight (*Ucp1*, r = –0.723, p = 0.004; *Pgc1a*, r = –0.541, p = 0.046) and blood glucose level (*Ucp1*, r = –0.700, p = 0.005; *Pgc1a*, r = –0.481, p = 0.082). Those of *Cdkn1a* and *Cdkn2a* in BAT were positively correlated with body weight (*Cdkn1a*, r = 0.760, p = 0.002; *Cdkn2a*, r = 0.787, p = 0.001) and blood glucose level (*Cdkn1a*, r = 0.537, p = 0.048; *Cdkn2a*, r = 0.662, p = 0.010; data not shown). These data suggest that greater adiposity and glycemia may have association with reduced thermogenesis and induced cellular senescence in BAT.

Adipogenic capability and cellular senescence of SVCs

To determine the effects of diabetes on adipogenesis and cellular senescence in APCs, the mRNA expression levels of related genes were measured in SVCs isolated from WAT and BAT (**Fig. 2**). The white SVCs of DB mice exhibited higher *Cebpa* expression, and their brown SVCs yielded higher *Pparg* and *Cebpa* expression levels than those of CON mice. Whereas the white SVCs of DB mice displayed lower *Cdkn2a* expression, their brown SVCs yielded higher *Cdkn1a* and *Cdkn2a* expression levels than those of CON mice.

Response of primary adipocytes to β-agonists

To substantiate whether diabetes modifies the thermogenic response and cellular senescence of adipocytes under β-adrenergic stimulation, NE or CL was administered to fully differentiated white and brown adipocytes, and the expression levels of related genes were measured (**Figs. 3** and **4**).

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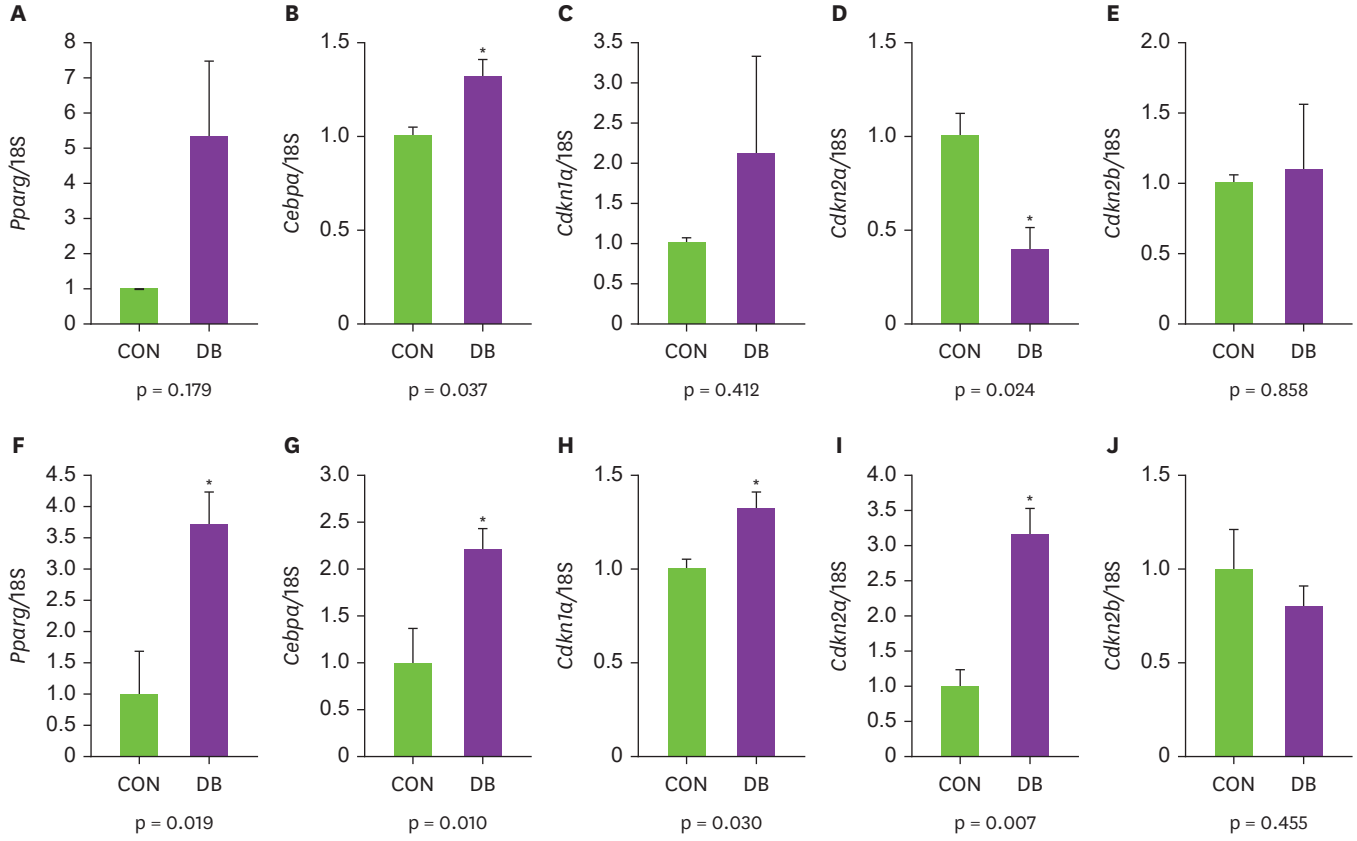

Fig. 2. Effects of diabetes on adipogenic and senescence gene expression in SVCs isolated from adipose tissues.
(A, B) Adipogenic genes in white SVCs, (C-E) senescence genes in white SVCs, (F, G) adipogenic genes in brown SVCs, and (H-J) senescence genes in brown SVCs. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 3, technical replicates).
CON, control; DB, *db/db*; SVC, stromal vascular cell.
*Indicate significant differences at $p < 0.05$ by Student's t-test.

Regardless of the β-agonist treatment, white adipocytes derived from DB mice yielded significantly lower thermogenic and cellular senescence gene expression levels than those derived from CON mice. NE upregulated *Pgc1a* expression in white adipocytes derived from CON mice, but not in those derived from DB mice. Both NE and CL suppressed *Prdm16* and *Cdkn2b* expression but induced *Cdkn1a* and *Cdkn2a* expression in CON mouse-derived white adipocytes (**Fig. 3**).

In brown adipocytes, irrespective of mouse genotype, NE downregulated *Prdm16* and *Cdkn2b* expression, and CL downregulated *Pgc1a*, *Prdm16*, and *Cdkn2b* expression. NE upregulated *Pgc1a* expression in brown adipocytes derived from CON mice, but not in those derived from DB mice. NE upregulated *Cdkn1a* expression, and CL upregulated *Cdkn2a* expression in DB mouse-derived brown adipocytes (**Fig. 4**).

Response of primary adipocytes to 18-carbon fatty acids

To verify whether diabetes alters the thermogenic response and cellular senescence of adipocytes under PPAR activation, 18-carbon fatty acids with varying quantities of double bonds, including STA, OLA, LNA, and ALA, were administered to fully differentiated white and brown adipocytes, and the expression levels of related genes were measured (**Figs. 5** and **6**).

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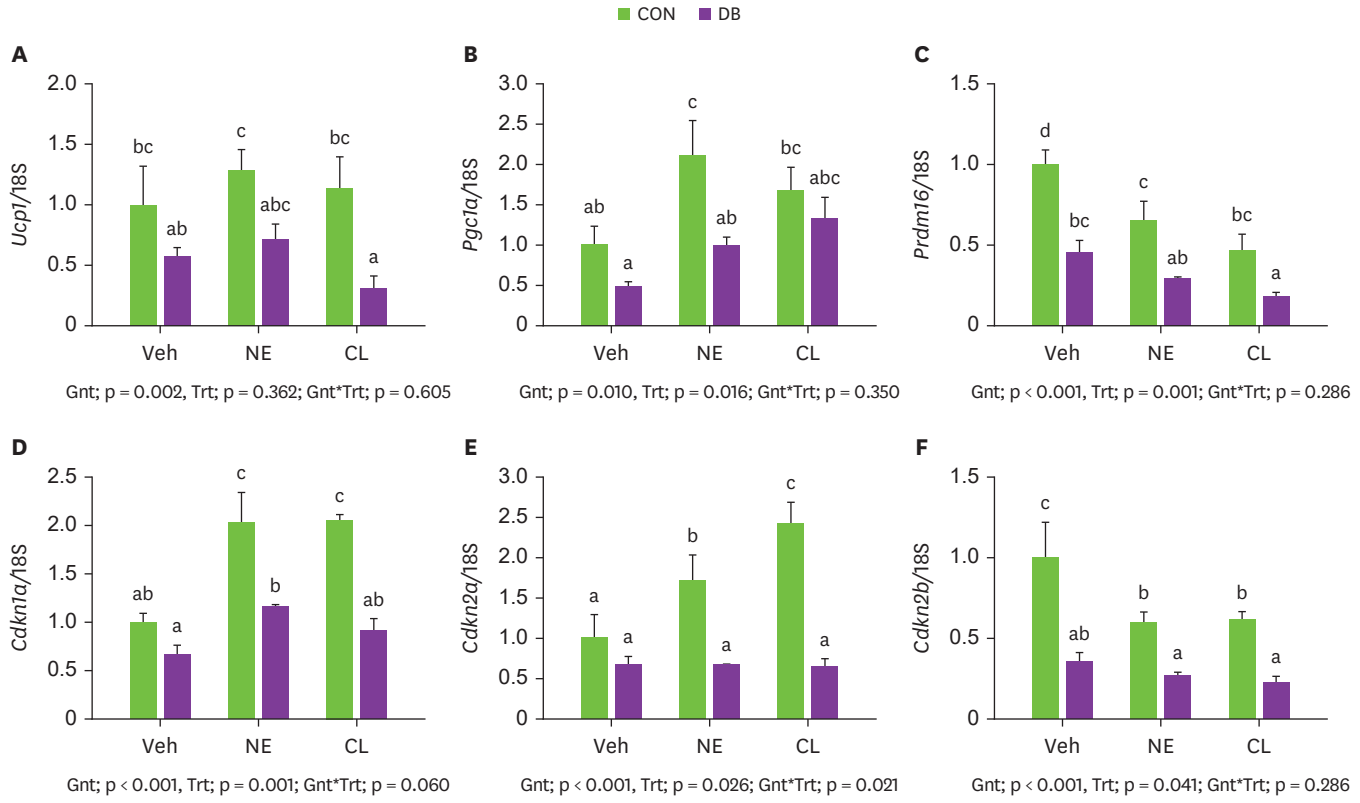

Fig. 3. Effects of diabetes on thermogenic and senescence gene expression in white adipocytes to β-agonists.
(A-C) Thermogenic genes and (D-F) senescence genes. Fully differentiated white adipocytes were treated with 10 μM of NE or CL for 4 hours. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 3, technical replicates).
CON, control; DB, *db/db*; Veh, vehicle; NE, norepinephrine; CL, CL316,243; Gnt, genotype effect; Trt, β-agonist treatment effect; Gnt*Trt, interaction effect.
[a-d]Different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's post-hoc test. If a group has a common letter with another group, it means they are not statistically different.

White adipocytes derived from DB mice yielded lower *Pgc1a*, *Prdm16*, *Cdkn2a*, and *Cdkn2b* expression levels and higher *Cdkn1a* expression than those derived from CON mice. However, the fatty acids within white adipocytes derived from the same genotype elicited no effect (**Fig. 5**). DB mouse-derived brown adipocytes yielded higher *Ucp1* and *Pgc1a* expression and lower *Cdkn2a* and *Cdkn2b* expression levels. Compared with OLA-treated brown adipocytes derived from DB mice, LNA-treated cells exhibited higher *Cdkn1a* and *Cdkn2b* expression levels (**Fig. 6**).

DISCUSSION

Although adipose tissue dysfunction is often accompanied by diabetes [7], the effects of diabetes on thermogenesis and cellular senescence in adipocytes remain unclear. Therefore, we examined the response of primary adipocytes isolated from DB mice, which exhibit key features of human type 2 diabetes (T2D) [21], in response to β-AR and PPAR activation.

In this study, DB mice displayed greater glycemia and adiposity than CON mice, as expected. This increased adiposity could be attributed to the upregulation of *Pparg* and/or *Cebpa* in SVCs isolated from adipose tissues. *Pparg* and *Cebpa* are known to regulate the terminal stage of

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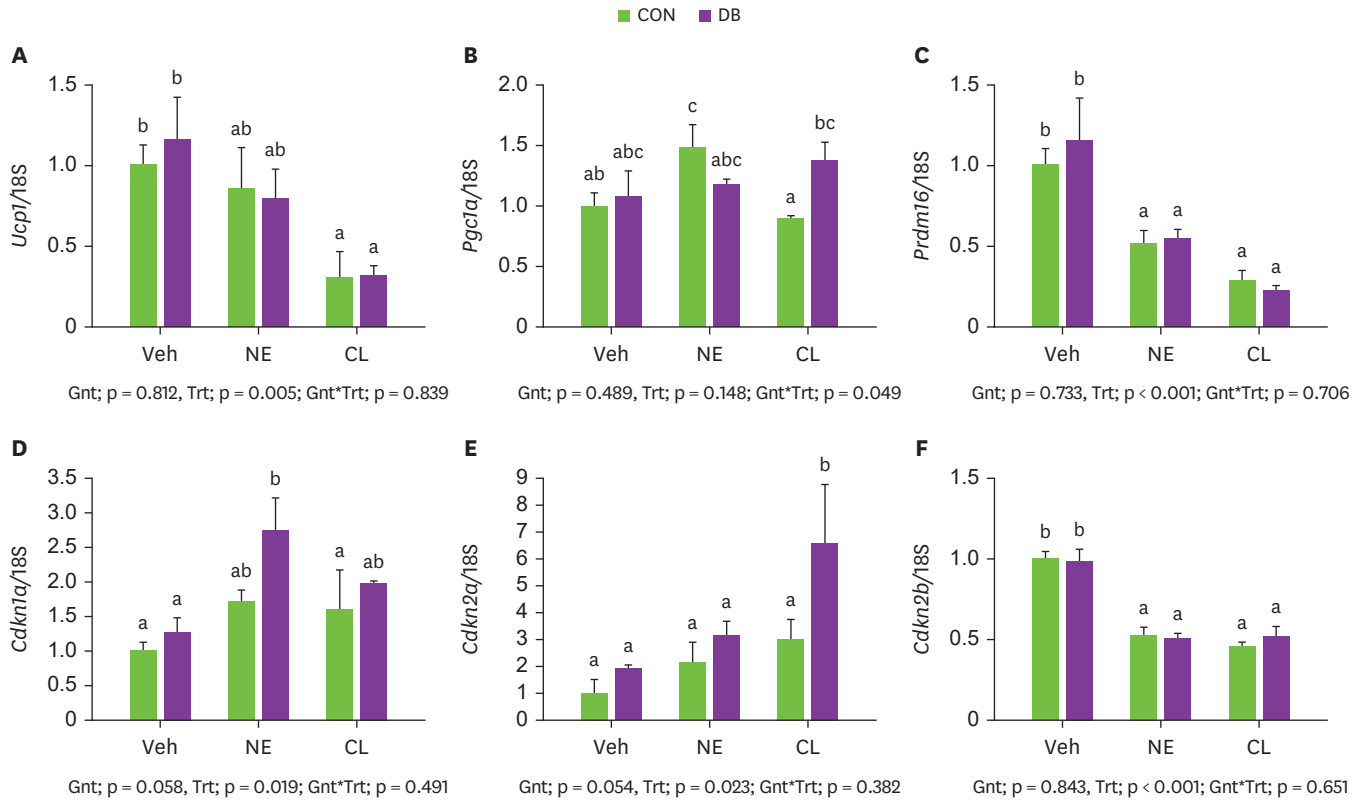

Fig. 4. Effects of diabetes on thermogenic and senescence gene expression in brown adipocytes to β-agonists.
(A-C) Thermogenic genes and (D-F) senescence genes. Fully differentiated brown adipocytes were treated with 10 μM of NE or CL for 4 hours. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 3, technical replicates).
CON, control; DB, *db/db*; Veh, vehicle; NE, norepinephrine; CL, CL316,243; Gnt, genotype effect; Trt, β-agonist treatment effect; Gnt*Trt, interaction effect.
[a-c]Different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's post-hoc test. If a group has a common letter with another group, it means they are not statistically different.

adipocyte differentiation, and their target genes induce lipid accumulation in adipocytes [16,22].

The greater BAT mass and lower *Ucp1* expression in DB mouse-derived BAT potentially indicate that diabetes induces BAT whitening. BAT whitening, that is the conversion of brown adipocytes to white-like adipocytes [23], is typically observed in obesity and promotes insulin resistance [16,23]. Consistent with our data, a T2D rat model was reported to have more fat deposits but lower UCP1 expression and fewer mitochondria in BAT [24]. Although DB mouse-derived WAT yielded numerically lower thermogenic gene expression values, no statistical significance was noted.

DB mice exhibited higher *Cdkn1a* and *Cdkn2a* expression levels in BAT, and these levels were positively correlated with body weight and blood glucose level. This may be because obesity and hyperglycemia accumulate senescent cells in adipose tissues [5], and the senescent immune cells that infiltrate BAT reportedly impair sympathetic innervation and thermogenic function [25]. *Cdkn1a* and *Cdkn2a* upregulation in DB mouse-isolated brown SVCs suggests that obesity and hyperglycemia may influence not only immune cells but also APCs in BAT.

Multiple types of cells in WAT also reportedly tend to become senescent in obesity or diabetes [13]; however, in our study, diabetes exerted no significant effect on WAT senescence.

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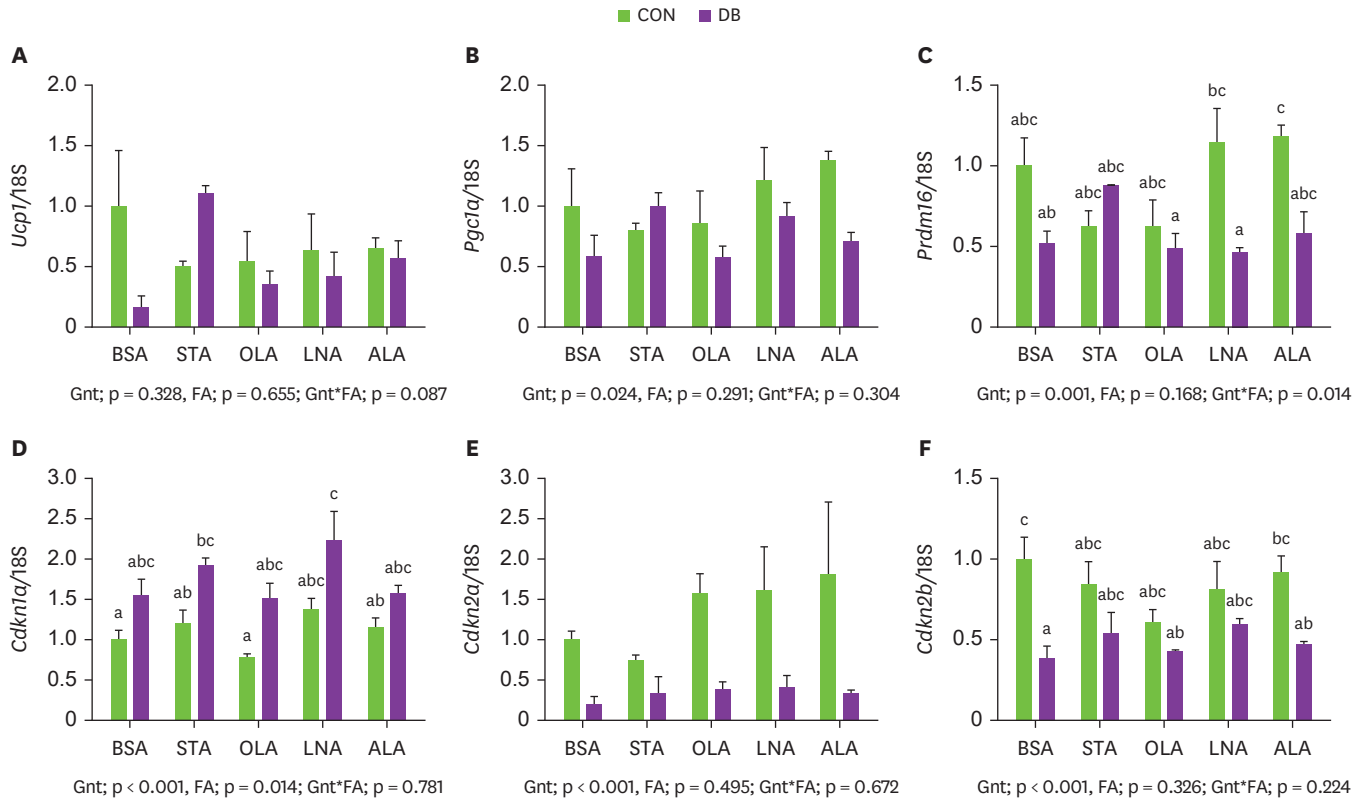

Fig. 5. Effects of diabetes on thermogenic and senescence gene expression in white adipocytes to 18-carbon fatty acids.
(A-C) Thermogenic genes and (D-F) senescence genes. Fully differentiated white adipocytes were treated with 50 μM of each fatty acid or BSA for 24 hours. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 3, technical replicates).
CON, control; DB, *db/db*; BSA, bovine serum albumin; STA, stearic acid; OLA, oleic acid; LNA, linoleic acid; ALA, α-linolenic acid; Gnt, genotype effect; FA, 18-carbon fatty acid effect; Gnt*FA, interaction effect.
[a-c]Different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's post-hoc test. If a group has a common letter with another group, it means they are not statistically different.

Instead, DB mouse-isolated white SVCs displayed lower *Cdkn2a* expression, and white mature adipocytes derived from these mice yielded lower *Cdkn2a* and *Cdkn2b* expression levels under basal conditions. In a study involving human participants with T2D and obesity, another key regulator of cellular senescence, *Cdkn2c* (also known as p18), was evidently downregulated in both subcutaneous and visceral WAT. *Cdkn2c* expression was negatively correlated with insulin resistance, and its deletion reduced fat accumulation in white adipocytes [26]. These data may suggest that diabetes downregulates the cellular senescence genes in white APCs and adipocytes, thus impairing lipid storage in adipocytes and exacerbating insulin resistance by promoting ectopic lipid storage, such as fatty liver.

Regardless of β-adrenergic stimulation, *Ucp1*, *Pgc1a*, and *Prdm16* expression was downregulated in fully differentiated white adipocytes derived from DB mice. Adipose tissue inflammation, induced by obesity or diabetes, is known to suppress whole-body thermogenesis by restraining brown-like phenotype acquisition of white adipocytes [22]. However, the thermogenic genes in BAT were not downregulated, indicating that BAT is more resistant to the suppression of thermogenesis induced by inflammation. Consistently, both HFD-induced chronic inflammation and lipopolysaccharide-induced acute inflammation have been reported to downregulate thermogenic genes and upregulate pro-inflammatory genes in the murine WAT, but not the BAT [18,19].

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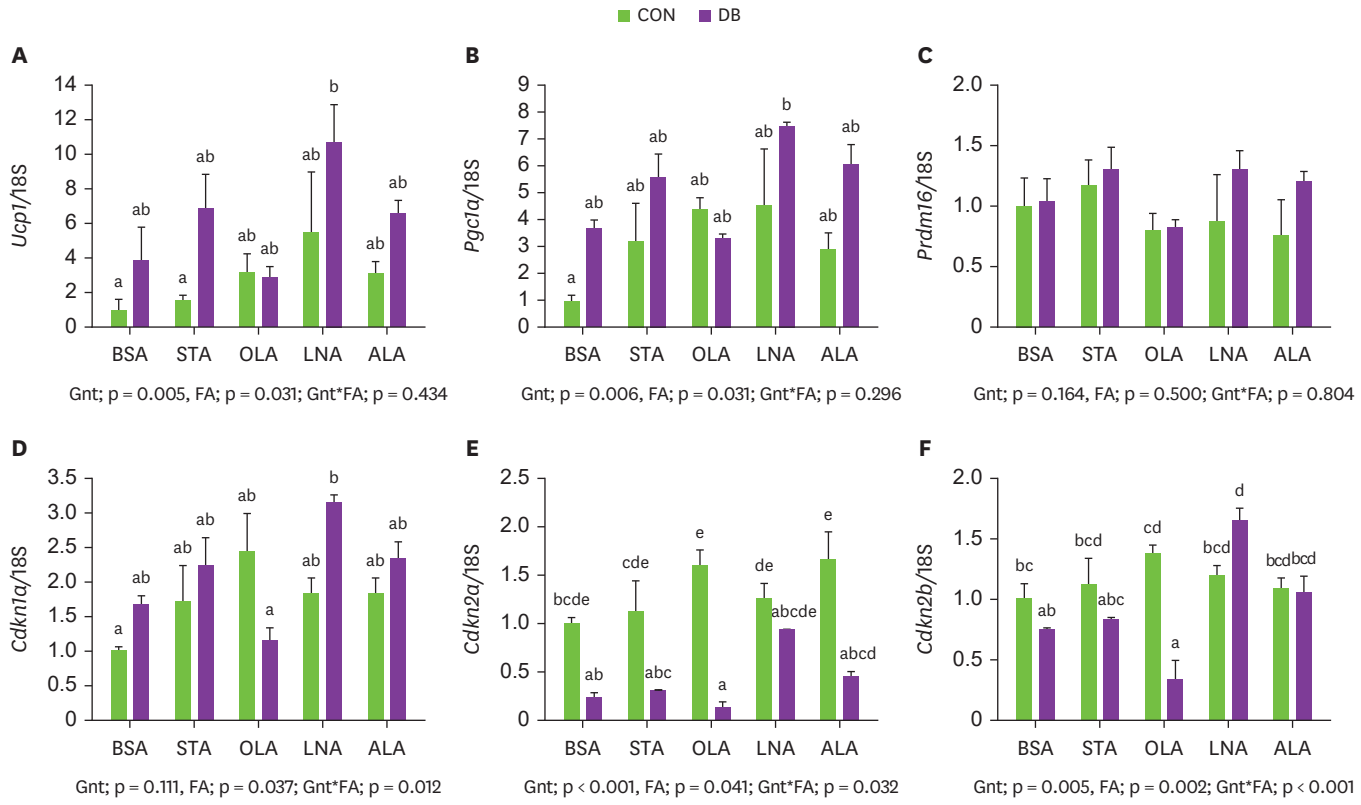

Fig. 6. Effects of diabetes on thermogenic and senescence gene expression in brown adipocytes to 18-carbon fatty acids.
(A-C) Thermogenic genes and (D-F) senescence genes. Fully differentiated brown adipocytes were treated with 50 μM of each fatty acid or BSA for 24 hours. The gene expression was determined by quantitative real-time polymerase chain reaction with normalization relative to 18S rRNA. Data are presented as means ± SEM (n = 3, technical replicates).
CON, control; DB, *db/db*; BSA, bovine serum albumin; STA, stearic acid; OLA, oleic acid; LNA, linoleic acid; ALA, α-linolenic acid; Gnt, genotype effect; FA, 18-carbon fatty acid effect; Gnt*FA, interaction effect.
[a-e]Different letters indicate significant differences at $p < 0.05$ by two-way ANOVA with Tukey's post-hoc test. If a group has a common letter with another group, it means they are not statistically different.

NE upregulated *Pgc1a* in both white and brown adipocytes derived from CON mice; however, it had no effect on this gene in cells derived from DB mice. This implies that diabetes may suppress the upregulation of the thermogenic response to β-adrenergic stimulation. Similar findings have been reported wherein, even with NE treatment, *Pgc1a* expression was lower in the visceral adipose tissue of obese mice than in that of lean mice [18] as well as in white primary adipocytes derived from HFD-fed mice than in those from low-fat diet-fed mice [20].

Both NE and CL treatment downregulated *Prdm16* expression in white and brown adipocytes regardless of mouse genotype, exhibiting negative feedback. Under β-adrenergic stimulation, PRDM16 and other transcription factors are recruited to upregulate thermogenic genes; therefore, to prevent overstimulation of the signaling, β-ARs are desensitized or downregulated [27]. Previous studies have reported that NE downregulates *Adrb3*, a β_3-AR encoding gene, in brown adipocytes [28] and mouse adipose tissues [18]. This also potentially explains the CL-induced downregulation of *Ucp1* in brown adipocytes derived from both genotypes.

Meanwhile, NE or CL upregulated *Cdkn1a* and/or *Cdkn2a* in CON mouse-derived white adipocytes and DB mouse-derived brown adipocytes, and this possibly emanated from the upregulation of lipolysis. The stimulation of β-adrenergic signaling is known to promote lipolysis by increasing the levels of intracellular cyclic adenosine monophosphate and

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activating cAMP-independent protein kinase (PKA). Perilipin-1 phosphorylated by PKA interacts with adipose triglyceride lipase (ATGL), which induces triglyceride hydrolysis [29]. Isoproterenol, another β-agonist, has been reported to upregulate *Cdkn1a* in mouse white fat pads, and this upregulation is reportedly reversed when ATGL is disrupted [30].

Our previous studies revealed the thermogenic effects of 18-carbon polyunsaturated fatty acids on primary adipocytes. In fully differentiated white adipocytes derived from subcutaneous fat of C57BL/6 mice, *Ucp1* and *Pgc1a* expression was induced by LNA and ALA treatment [17]. In this study, these fatty acids had no effect on thermogenic gene expression in both white and brown adipocytes. In addition, compared with the BSA control, no fatty acids were found to downregulate cellular senescence genes in the adipocytes. However, OLA-treated brown adipocytes derived from DB mice yielded lower *Cdkn1a* and *Cdkn2b* expression levels than LNA-treated cells. This may be related to reactive oxygen species (ROS), which induce and maintain the senescent phenotype [31]. In multiple types of fibroblasts, LNA has been reported to stimulate ROS production more than OLA [32].

A limitation of this study is that the expression levels of adipogenic, thermogenic, and cellular senescence genes were exclusively determined at the mRNA levels. Nevertheless, this study provides novel evidence regarding the association between adipose thermogenesis and cellular senescence in diabetes under basal conditions and in response to stimuli. Moreover, multiple studies have reported that the mRNA expression of the genes assessed in the current study is consistent with the phenotype [8,9,11].

SUMMARY

Taken together, diabetes suppresses *Ucp1* expression in BAT and the NE-induced upregulation of *Pgc1a* in both white and brown adipocytes. It also upregulates *Cdkn1a* and *Cdkn2a* expression in BAT, and OLA-treated brown adipocytes derived from DB mice exhibit a lower expression of these genes than LNA-treated cells. These data imply that adipose thermogenesis may be inversely associated with cellular senescence in diabetes, and different types of fatty acids potentially exert divergent effects on adipose thermogenesis and cellular senescence.

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