

Apelin Enhances Brown Adipogenesis and Browning of White Adipocytes*

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Background: The autocrine regulatory effects of apelin on self-remodeling of adipose tissue are not known.

Results: Apelin enhances not only the differentiation and metabolic activity of brown adipocytes but also the browning of white adipocytes.

Conclusion: Apelin-APJ signaling promotes adipose tissue browning.

Significance: Apelin signaling may serve as a potential therapeutic target for obesity and associated metabolic diseases.

Brown adipose tissue expends energy in the form of heat via the mitochondrial uncoupling protein UCP1. Recent studies showed that brown adipose tissue is present in adult humans and may be exploited for its anti-obesity and anti-diabetes actions. Apelin is an adipocyte-derived hormone that plays important roles in energy metabolism. Here, we report that apelin-APJ signaling promotes brown adipocyte differentiation by increasing the expressions of brown adipogenic and thermogenic transcriptional factors via the PI3K/Akt and AMPK signaling pathways. It is also found that apelin relieves the TNF α inhibition on brown adipogenesis. In addition, apelin increases the basal activity of brown adipocytes, as evidenced by the increased PGC1 α and UCP1 expressions, mitochondrial biogenesis, and oxygen consumption. Finally, we provide both *in vitro* and *in vivo* evidence that apelin is able to increase the brown-like characteristics in white adipocytes. This study, for the first time, reveals the brown adipogenic and browning effects of apelin and suggests a potential therapeutic route to combat obesity and related metabolic disorders.

Brown adipose tissue (BAT)² is the primary site for non-shivering thermogenesis in response to cold exposure, especially in newborns, through the actions of heat-producing brown fat cells (1, 2). Mediated by mitochondrial uncoupling

protein 1 (UCP1), brown adipocytes have the ability to uncouple oxidative metabolism of energy substrates (glucose and fat) from ATP synthesis, leading to heat production and energy consumption (1). Recent studies have demonstrated that metabolically active BAT, which was thought to disappear after infancy, is present in adult humans and is inversely correlated with body mass index and visceral fat mass (2–4). Studies have shown that an increased amount of BAT is associated with weight loss in obese subjects, and increased brown fat cell differentiation leads to an increase in energy consumption and reduction in body weight gain and diet-induced obesity (5–7). BAT also regulates glucose homeostasis and improves insulin sensitivity (8, 9), and brown adipogenesis is suppressed in human subjects with insulin resistance (10). Hence, stimulating brown fat formation could be a novel route to fight against obesity and related metabolic disorders (11).

In contrast, excess accumulation of white adipose tissue (WAT) has notorious effects on the development of metabolic disorders, such as diabetes and cardiovascular diseases (12). Interestingly, brown fat-like cells (also known as beige cells) have been identified in WAT, and brown fat-like characteristics (*e.g.* UCP1 expression) are reduced in WAT of obese humans (13). Studies have demonstrated that an increase of beige adipocytes in WAT enhances whole-body energy expenditure and should expectedly reduce the risk of diet-induced obesity and metabolic diseases (14, 15). Differentiation of white adipocytes into beige cells occurs in response to prolonged cold exposure and can be stimulated by hormones such as β -adrenergic receptor agonists, irisin, thyroid hormone, fibroblast growth factor 21 (FGF21), etc. (15–17). On the other hand, it is increasingly recognized that WAT is not merely a passive depot for storage of excess energy as fat but also the largest endocrine organ, producing a variety of bioactive factors called adipokines (18). These adipokines play essential roles in regulating energy metabolism and also influence adipose tissue's own remodeling and functions (18–20). Emerging evidence also suggests that adipokines are important for adipose browning. For example, adiponectin and leptin modulate UCP1 expression in rodent BAT (21, 22), whereas interleukin-6 is required for cold-induced UCP1 expression in WAT of mice (23).

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² The abbreviations used are: BAT, brown adipose tissue; WAT, white adipose tissue; Akt, protein kinase B; AMPK, 5'-adenosine monophosphate-activated protein kinase; APJ, apelin receptor; AR, adrenergic receptor; aP2, adipocyte protein 2 (adipocyte-fatty acid-binding protein); COX1, cytochrome c oxidase subunit I; CIDE-A, cell death-inducing DFFA-like effector A; C/EBP β , CCAAT-enhancer binding protein β ; PPAR γ , peroxisome proliferator-activated receptor γ ; PRDM16, PR domain-containing protein 16; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1- α ; NE, norepinephrine; OCR, oxygen consumption rate; UCP1, uncoupling protein 1; hMSCs, human mesenchymal stem cells; ANOVA, analysis of variance.

Apelin Stimulates Browning of Adipose Tissue

Apelin is a relatively recent member of the adipokine family (24). Apelin regulates various aspects of energy metabolism through APJ receptors and has been known for its anti-obesity and anti-diabetic properties (25). Apelin promotes insulin sensitivity and glucose utilization in adipose and muscle tissues (26, 27). Apelin injection decreases WAT mass and serum triglyceride level in obese mice, whereas apelin knock-out mice have augmented body adiposity and serum free fatty acid levels (28). Apelin also suppresses white adipocyte differentiation and lipolysis (28, 29). The antagonistic effects of apelin on diet-induced obesity and insulin resistance are attributable to its stimulation on UCP1 expression in BAT and body energy expenditure (30, 31). Because apelin production in adipocytes and its plasma level are elevated in obesity (24), we speculate that apelin may provide a beneficial feedback control on both brown and white adipocytes. Here, we aim for the first time to reveal the effects of apelin on adipose browning (differentiation of brown preadipocytes in BAT, metabolic activities of mature brown adipocytes, and browning of white adipocytes in WAT).

Experimental Procedures

Primary Preadipocyte Culture and Differentiation—The primary rat brown preadipocytes isolated from interscapular BAT were purchased from CosmoBio Co. Ltd. (Tokyo, Japan). In some experiments, the primary white and brown preadipocytes, isolated from mouse WAT and interscapular BAT, were also used. After digestion with 0.2% collagenase and centrifugation (1,500 rpm, 5 min), the stromal vascular fraction/preadipose cells from WAT and BAT were isolated as described before (32). Preadipose cells were seeded in 6-well plates and cultured with the basal growth medium (DMEM supplemented with 10% fetal bovine serum (FBS), 1 nM 3,5,3'-triiodothyronine, 1% penicillin and streptomycin) at 37 °C and in a humidified atmosphere containing 5% CO₂ and 95% air. For adipocyte differentiation, fully confluent cells (defined as day 0) were treated for 2 days with induction medium (basal growth medium supplemented with 0.5 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine). At day 2, the cells were incubated for another 2 days with growth medium containing insulin, and thereafter, the medium was replenished every other day for the next 6–8 days. The Transwell indirect contact co-culture system (Corning Inc.) was used to co-culture the primary rat brown preadipocytes and primary rat macrophages (isolated from adult rat bone marrow tissue; ScienCell Research Laboratories).

The human preadipocytes (Zen-Bio Inc.), collected from subcutaneous WAT (non-diabetic subject, body mass index 25–29.9), were cultured in DMEM/F-12 (1:1, v/v) containing 10% FBS until confluence. Adipocyte differentiation was then induced similarly as reported previously (33, 34). Specifically, the cells were treated for 4 days with human adipocyte growth medium (serum-free DMEM/F-12 containing 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 1 nM 3,5,3'-triiodothyronine) supplemented with 0.5 μg/ml insulin, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 1 μM troglitazone. The cells were then incubated in the growth medium containing insulin and dexamethasone for 12–14 days (replenishment every other day). The mouse 3T3-L1 cells (white preadipose cell

line, ATCC) were cultured in DMEM supplemented with 10% bovine calf serum until confluence. The cells were then treated with the induction medium for 2 days (days 0–2), followed by growing for an additional 2 days in the growth medium containing insulin alone (days 2–4). Finally, the cells were grown for 4–6 days without insulin.

The human mesenchymal stem cells (hMSCs; Lonza Inc.) collected from bone marrow of normal subjects were grown in the basal growth medium until confluence. The stem cells were subsequently induced to differentiate into adipocytes as described previously (35). Specifically, stem cells were treated for 3 days in the induction medium (plus 200 μM indomethacin). In the next 12–14 days, the cells were maintained in the growth medium containing insulin alone (replenishment every other day). Adipocyte differentiation was confirmed by Oil Red O staining, visual appearance of intracellular lipid droplets, and immunoblot analyses of adipocyte-specific proteins (e.g. adipocyte protein 2 (ap2), leptin) as well as brown adipocyte-specific proteins (e.g. UCP1). At least two different batches (donors) of the primary cells were used for every experiment. All culture media, supplements, and sera were purchased from Life Technologies, Inc. Pyr-apelin13 (pyroglutamated 13-amino acid peptide), insulin, and troglitazone were purchased from Tocris Bioscience. All other reagents and chemicals were obtained from Sigma-Aldrich.

Animal Studies—All animal experimental protocols were approved by the SingHealth Research Facilities Institutional Animal Care and Use Committee (Singapore). Mice (C57BL/6J, 8–10 weeks old, male) were housed in light- and temperature-controlled facility (12-h light/12-h dark cycle, 21 °C), and allowed free access to standard laboratory food and water. Mice were divided into apelin-treated and non-treated (100 μl of saline, control) groups. Based on the previous studies (30, 31), apelin was given at a dose of 0.1 μmol/kg/day (dissolved in 100 μl of saline) by intraperitoneal injection for 12 days. Animals were sacrificed 24 h after the last dose, and body and fat tissue weights were measured in all animals. Inguinal WAT, epididymal WAT, and interscapular BAT were removed and frozen in liquid nitrogen for further experiments. Tissues were homogenized in radioimmunoprecipitation assay buffer (containing protease inhibitor mixture) (Roche Applied Science), and protein concentration was determined by a bicinchoninic acid (BCA) protein assay (Pierce) before the immunoblot analyses were carried out (using the same amount of the protein lysates).

siRNA Silencing—Gene silencing of apelin (*APLN*) or APJ receptor (*APLNR*) in brown preadipocytes was achieved using a rat *APLN* siRNA or *APLNR* siRNA (Ambion Silencer Select, Life Technology), respectively. Akt siRNAs (sc-43610 and sc-108059; Santa Cruz Biotechnology, Inc.) and AMPK siRNAs (sc-45313 and sc-270142) were also used for gene silencing of Akt or AMPK in adipose cells. Transfection was done as described previously (33). Specifically, adipose cells were incubated with Opti-MEM containing a complex of Lipofectamine-RNAiMAX transfection reagent (0.5% (v/v), Life Technologies, Inc.) with siRNAs (25 nM) for 4 h, followed by the addition of basal growth medium and incubation for another 1–2 days. Knockdowns of protein expression were confirmed by immunoblot analyses.

Confocal Microscopy—Adipocytes grown on Lab-Tek II chambered coverglass were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and then washed twice with ice-cold PBS. In some experiments, the cells were permeabilized with 0.1% Triton X-100 for 10 min. After being blocked with 1% bovine albumin (BSA) in PBST (PBS with 0.1% Tween 20) at room temperature for 1 h, the cells were then incubated (4 °C) overnight in PBST with 1% BSA and primary antibodies (Santa Cruz Biotechnology): anti-apelin IgG (sc-33805), anti-APJ IgG (sc-33823), anti-UCP1 IgG (sc-6529), anti- β 1-AR IgG (sc-568), or anti β 3-AR IgG (sc-50436). After washing with PBS, the cells were incubated in FITC-labeled or Atto647 NHS-labeled secondary antibody (Sigma) for 1 h at room temperature, followed by washing three times with PBS. Adipocytes were then imaged using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss GmbH). To reveal the mitochondrial membrane potential and morphology, adipocytes were incubated with the growth medium containing 0.2 μ M MitoTracker Red FM (Life Technologies) (excitation/emission, 581 nm/644 nm) (at 37 °C with 5% CO₂ in a humidified incubator) for 15 or 30 min, before live cell imaging with a confocal microscopy. MitoTracker Red is widely used to assess both mitochondrial morphology and membrane potential in living cells, because it is selectively localized in mitochondria, whereas its accumulation is dependent on transmembrane potential (34, 36–38).

Western Blot Analyses—The cells were washed with ice-cold PBS and collected in mammalian protein extraction reagent containing Halt protease and phosphatase inhibitor mixture (Fisher). After brief vortexing and centrifugation at 4 °C, the supernatant was collected, and the protein content was determined by a BCA protein assay (Pierce). Each sample with an equal amount of proteins was separated through 12% SDS-PAGE before it was transferred onto a nitrocellulose membrane. The membranes were then blocked for 2 h at room temperature in Superblock blocking buffer, followed by subsequent incubation for 12 h in Tris-buffered saline-Tween solution (TBST) containing primary antibody (1:100–400 dilution). After washing three times with TBST, the membrane was incubated for 6 h with horseradish peroxidase-conjugated secondary antibody (1:2,000–4,000). The protein bands were detected in a G:BOX Chemi XT4 imaging system (Syngene) using Super-Signal West Pico chemiluminescent substrate (Fisher). Antibodies against apelin (sc-33469), APJ (sc-33823), UCP1 (sc-6529), CIDE-A (sc-366814), aP2 (sc-18661), COX1 (sc-23982), C/EBP β (sc-150), leptin (Ob, sc-9014), PPAR γ (sc-7196), PGC-1 α (sc-13067), PRDM16 (sc-55697), β 1-AR (sc-568), β 3-AR (sc-50436), Akt (sc-8312), phospho-Akt (Ser-474, sc-135651), AMPK α 1/2 (sc-25792), or phospho-AMPK α 1/2 (Thr-172; sc-33524) were purchased from Santa Cruz Biotechnology. Antibodies against UCP1 (PA1–24894) or PRDM16 (PA5–20872) from Fisher were also used in our experiments.

Oxygen Consumption Rate Assay—Oxygen consumption was assessed using an oxygen consumption rate assay kit (MitoXpress-Xtra HS method) (Cayman Chemical) according to the manufacturer's instructions. Briefly, preadipocytes were grown in 96-well plates (3 \times 10⁴ cells/well) until confluence and induced to differentiate into adipocytes. Adipocytes were treated with 10 μ l of MitoXpress-Xtra (a phosphorescent oxy-

gen-sensitive probe whose signal increases over time when O₂ is depleted in the solution) immediately before measuring the fluorescence intensity (excitation/emission, 380 nm/650 nm at 37 °C, 0.5-min interval) using a time-resolved fluorescence plate reader (SpectraMax M5, Molecular Devices). Measurement was performed under a sealed environment (by overlaying each well with HS Mineral Oil), in which the exchange of O₂ was limited. Oxygen-consuming glucose oxidase was used as a reference.

Statistical Analyses—Data were analyzed as mean \pm S.E. Statistical differences between two means were assessed using Student's *t* test (unpaired, two-tailed). One-way analysis of variance (ANOVA) (followed by Tukey's multiple-comparison test) was used to compare differences among multiple groups. A *p* value of <0.05 was considered to be statistically significant.

Results

Apelin-APJ Receptor Promotes Brown Adipocyte Differentiation—Apelin/APJ system is present in BAT (39). Here, we found that both apelin and APJ receptor are expressed in brown preadipocytes and adipocytes (Fig. 1, *A* and *H*). As shown in Fig. 1, *B* and *C*, exogenous application of apelin (since the induction of differentiation at day 0) significantly increased the number of differentiated brown adipocytes indicated by their multilocular appearance. Pyr-apelin13 (referred to as apelin) was used because it is the most active and major apelin isoform enzymatically cleaved from preproapelin (40, 41). Consistently, apelin also promoted the progressive expressions of UCP1 (thermogenesis marker), CIDE-A (brown adipocyte-specific marker), and aP2 (late marker of differentiated adipocyte) (Fig. 1, *D–G*) during brown adipogenesis.

However, apelin applied at the later stages of differentiation (at day 2 or 4) failed to increase the number of brown adipocytes (Fig. 1*C*), suggesting the importance of apelin signaling in the initiation of brown adipogenesis. As shown in Fig. 1*H*, after the initial burst increase (first day), preproapelin expression remained steady during brown adipogenesis, and the initial increase of preproapelin can be largely enhanced by exogenous apelin, suggesting that apelin may serve as the positive feedback signal to initiation of differentiation. In support of this notion, the number of mature brown adipocytes as well as expressions of UCP1, CIDE-A, and aP2 are considerably reduced when apelin and APJ in brown preadipocytes were knocked down by siRNAs (Fig. 1, *I–M*). Taken together, we provide evidence that apelin-APJ promotes brown adipocyte differentiation through autocrine feedback regulation.

Apelin Remedies Inflammation-impaired Brown Adipogenesis—BAT mass is lower in obese humans (3, 4). Studies also suggest that adipose tissue inflammation (as characterized by accumulation of macrophages and inflammatory cytokines, such as TNF α) found in obesity may be responsible for reduced brown fat by inhibiting differentiation and inducing apoptosis of brown adipocytes (42–45). To further investigate this, indirect co-culturing of brown preadipocytes and macrophages (primary rat macrophages) was performed (Fig. 2*A*) (46). As shown in Fig. 2*B*, co-culturing with macrophages (days 0–2) significantly decreased the expressions of UCP1, CIDE-A, and aP2 in brown fat cells (at day 10), implying that inflammatory

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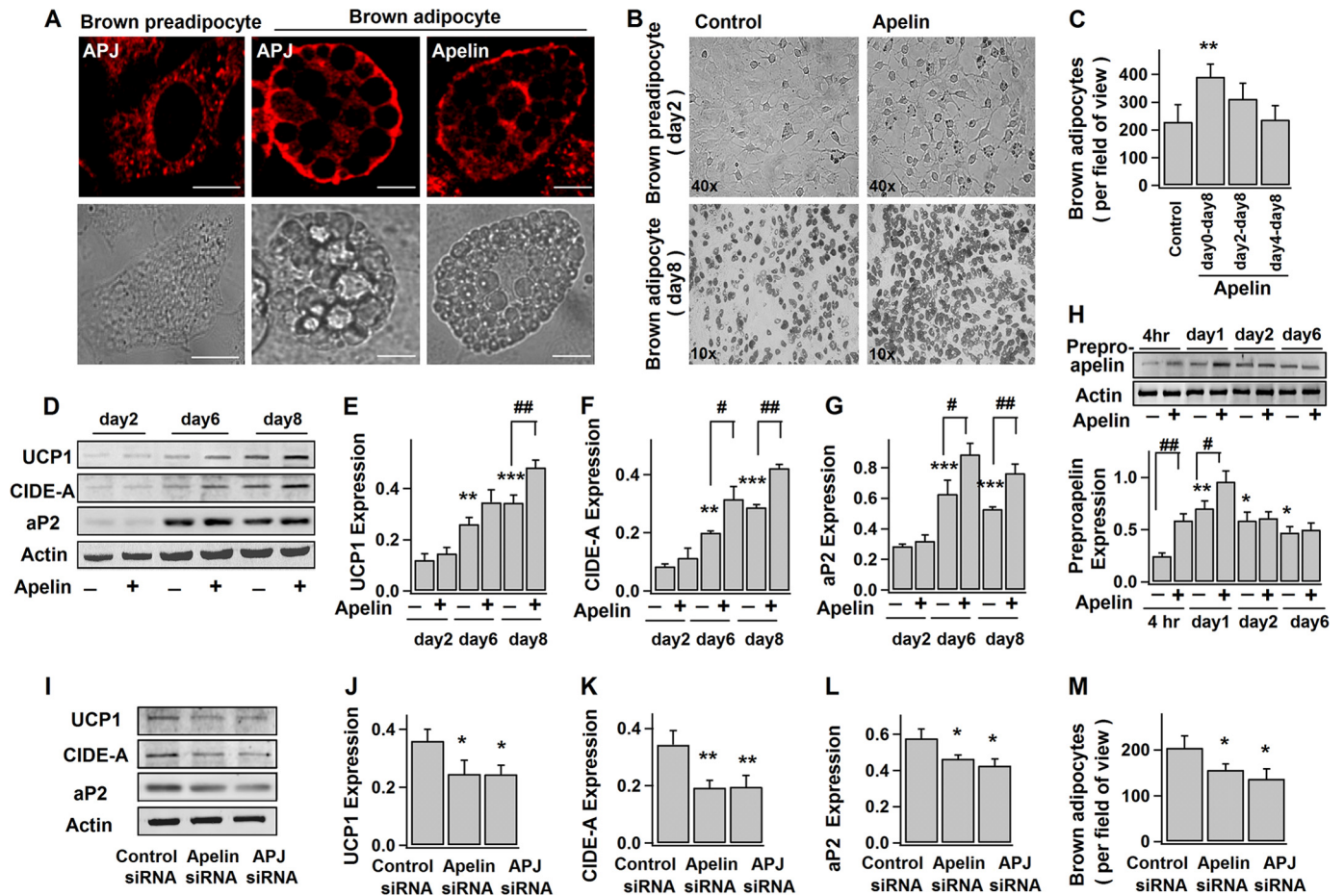


FIGURE 1. Apelin-APJ receptor enhances brown adipocyte differentiation. *A*, confocal images of immunostained apelin and APJ receptor in brown fat cells and their corresponding bright field images. *Scale bars*, 10 μ m. *B–H*, brown preadipocytes were induced to differentiate into adipocytes without (–) (control) or (+) with exposure to 100 nM pyr-apelin13 for different periods of time. *B*, representative images of brown preadipocytes (day 2) and adipocytes (day 8). *C*, number of brown adipocytes (multiloculated cells) (at day 8) per field of view ($\times 10$); mean \pm S.E. (*error bars*) ($n = 4$). *D–G*, time course of the protein expression levels during adipogenesis: UCP1 (~33 kDa), CIDE-A (~26 kDa), aP2 (~16 kDa), and actin (~42 kDa). The representative immunoblots and the statistics (mean \pm S.E., $n = 4$) of the blot densities normalized to actin density (protein expressions at day 2 regarded as control). *H*, representative immunoblot of preproapelin (~8 kDa) expression in brown fat cells at different stages of differentiation and the statistics (mean \pm S.E., $n = 4$; blot densities were normalized to that of actin) (expression at 4 h as control). *I–M*, brown preadipocytes were transfected with control, apelin, or APJ siRNA 2–3 days before the induction of differentiation. The representative immunoblot of protein expressions (day 10) and the statistics (mean \pm S.E., $n = 4$; blot densities were normalized to that of actin) are shown in *I–L*. The number of brown adipocytes (day 8) per field of view ($\times 10$) (mean \pm S.E., $n = 4$) is shown in *M*. Each sample contains the same amount of total proteins (as the loading control). Using Student's *t* test, *, $p < 0.05$; **, $p < 0.01$ versus control; #, $p < 0.05$; ##, $p < 0.01$ between the indicated pairs.

factors released from macrophages antagonize brown adipocyte differentiation. This is supported by the observation that direct application of TNF α (major inflammatory factor released from macrophages) reduced the number of brown adipocytes and expressions of brown adipocyte markers (Fig. 2, C–E). Interestingly, apelin was able to relieve the inhibitory effects of macrophages and TNF α (Fig. 2, B–E). Together with the fact that apelin expression in adipose tissue and plasma apelin level are elevated in obesity (24), our results suggest a compensatory increase in apelin to inhibited brown adipogenesis by adipose inflammation.

Apelin Stimulates Expressions of Brown Adipogenic and Thermogenic Transcriptional Factors—A number of transcriptional factors govern the process of adipocyte differentiation and terminal determination of brown adipocytes (e.g. UCP1 expression) (47). Among them, C/EBP β and PPAR γ are critical to initiate both white and brown adipogenesis (47, 48), whereas PRDM16 and PGC1 α are essential to brown fat determination

by stimulating expressions of thermogenic and brown-specific genes and repressing the expression of white-specific genes (49–51). Here, we observed that PPAR γ and PGC1 α exhibited a progressive increase of expression during differentiation, whereas C/EBP β and PRDM16 levels were steady (Fig. 3, A–E). This is consistent with previous reports (49, 52). Notably, apelin treatment increased the expressions of C/EBP β , PPAR γ , and PGC1 α in the early stage of differentiation and up-regulated the PRDM16 level throughout the differentiation period (Fig. 3). This is in line with the findings shown in Figs. 1 and 2 that apelin increases the number of differentiated brown adipocytes as well as the expressions of brown fat proteins required for thermogenesis and mitochondrial biogenesis (UCP1, CIDE-A, and COX1).

PPAR γ is well described as an important regulator of brown adipocyte differentiation (48). However, recent studies have suggested that PPAR γ does not control the terminal determination of brown adipocytes because PPAR γ overexpression in

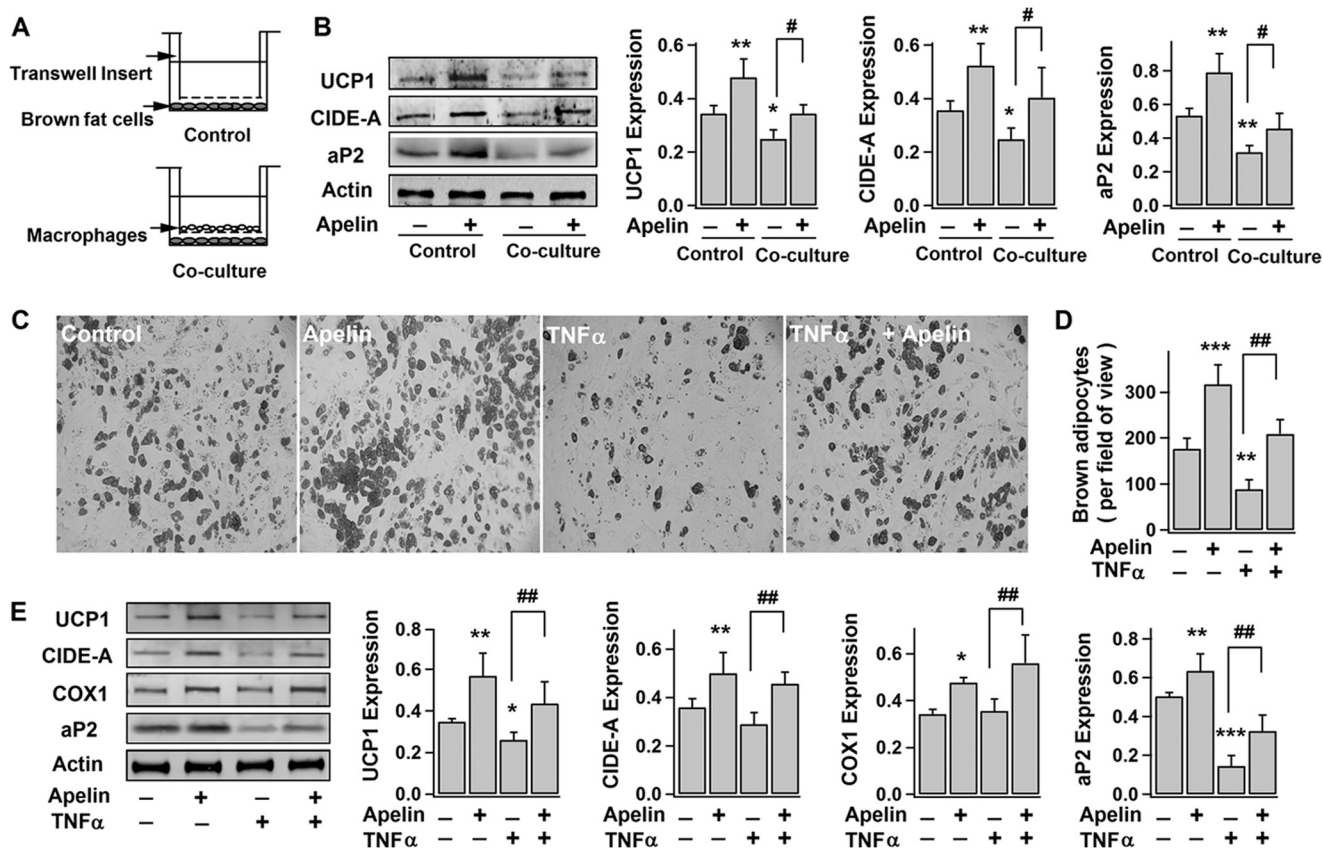


FIGURE 2. Apelin improves TNF α - and macrophage-impaired brown adipocyte differentiation. A and B, brown preadipocytes were co-cultured with primary rat macrophages during the first 2 days of differentiation, followed by continuing differentiation for 8 days. A, illustration of Transwell co-culturing system. The representative immunoblots of protein expressions (UCP1, CIDE-A, aP2, actin) (day 10) in brown adipocytes and the statistics (mean \pm S.E. (error bars), $n = 4$) of blot densities normalized to that of actin are shown in B. C–E, brown preadipocytes were induced to differentiate into brown adipocytes for 8–10 days, without (–) (control) or with (+) exposure to pyr-apelin13 and/or 10 ng/ml TNF α . Shown are representative bright field images (C) and the number of brown adipocytes (at day 8) per field of view ($\times 10$) (mean \pm S.E., $n = 4$) (D). The representative immunoblots of UCP1, CIDE-A, COX1 (~57 kDa), aP2, and actin expressions (day 10) and the statistics (mean \pm S.E., $n = 4$) of blot densities normalized to that of actin are shown in E. Using one-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control (no treatment); #, $p < 0.05$; ##, $p < 0.01$ between the indicated pairs.

hMSCs only induces white (not brown) fat phenotype (53); UCP1 expression was not suppressed in brown adipocytes of PPAR γ -ablated mice (54); and high dose PPAR γ agonist increased whitening of brown adipocyte (as indicated by enhanced lipogenesis, decreased UCP1 expression, and enlarged adipocytes with high lipid content and droplet size) (55, 56). In line with these previous reports, long term treatment with PPAR γ agonist (troglitazone, 10 μ M, days 2–10) induced more enlarged adipocytes with pale color and large intracellular lipid droplets (probably due to whitening of differentiated brown adipocytes; Fig. 3F).

Consistently, PPAR γ agonist stimulated the expression of aP2 (adipocyte differentiation marker) to a much greater extent as compared with the simulated expressions of UCP1 and CIDE-A (brown adipocyte-specific markers) (Fig. 3, G–M). We observed that apelin only stimulated PPAR γ expression at the early stage (within 2 days; Fig. 3E), and apelin co-treatment with PPAR γ agonist (since day 2) significantly increased the expressions of UCP1 and CIDE-A and the number of dark color brown adipocytes. In contrast, apelin was not able to rectify the inhibited brown differentiation by PPAR γ antagonist (T0070907, 1 μ M) applied in the first 2 days (data not shown). Taken together, apelin promotes brown adipogenesis by 1) stimulating expression of adipogenic transcriptional factors (C/EBP β and PPAR γ)

at the early stage and 2) up-regulating the transcription factors (PRDM16 and PGC1 α) required for thermogenesis and mitochondrial biogenesis and counteracting PPAR γ effects in the later stage to ensure terminal determination of the brown phenotype.

Apelin Raises the Basal Metabolic Activity of Brown Adipocytes—Norepinephrine (NE) released from the sympathetic nervous system activates brown adipocytes (including UCP1 expression, mitochondrial biogenesis, and respiratory activity) through β -adrenergic receptors (β -ARs), particularly β 3-AR (1). In agreement with previous studies (14), NE (6-h incubation) acutely promoted the expressions of PGC1 α (a key regulator of thermogenic gene expression and mitochondrial biogenesis), COX1, and UCP1 as well as the oxygen consumption rate (OCR), which reflects mitochondrial respiratory activity and metabolic rate, in brown adipocytes (Fig. 4, A–C). Similar to the NE effects, long term treatment with apelin (3–4 days) increased the expressions of PGC1 α , COX1, and UCP1 in brown adipocytes (Fig. 4, A and B). Significant up-regulation of β 3-AR, but not β 1-AR, was also observed (Fig. 4, E and F). As shown in Fig. 4, C and D, apelin was able to stimulate OCR to a level comparable with acute stimulation by NE. However, apelin pretreatment had little influence on NE-induced protein expressions and OCR (Fig.

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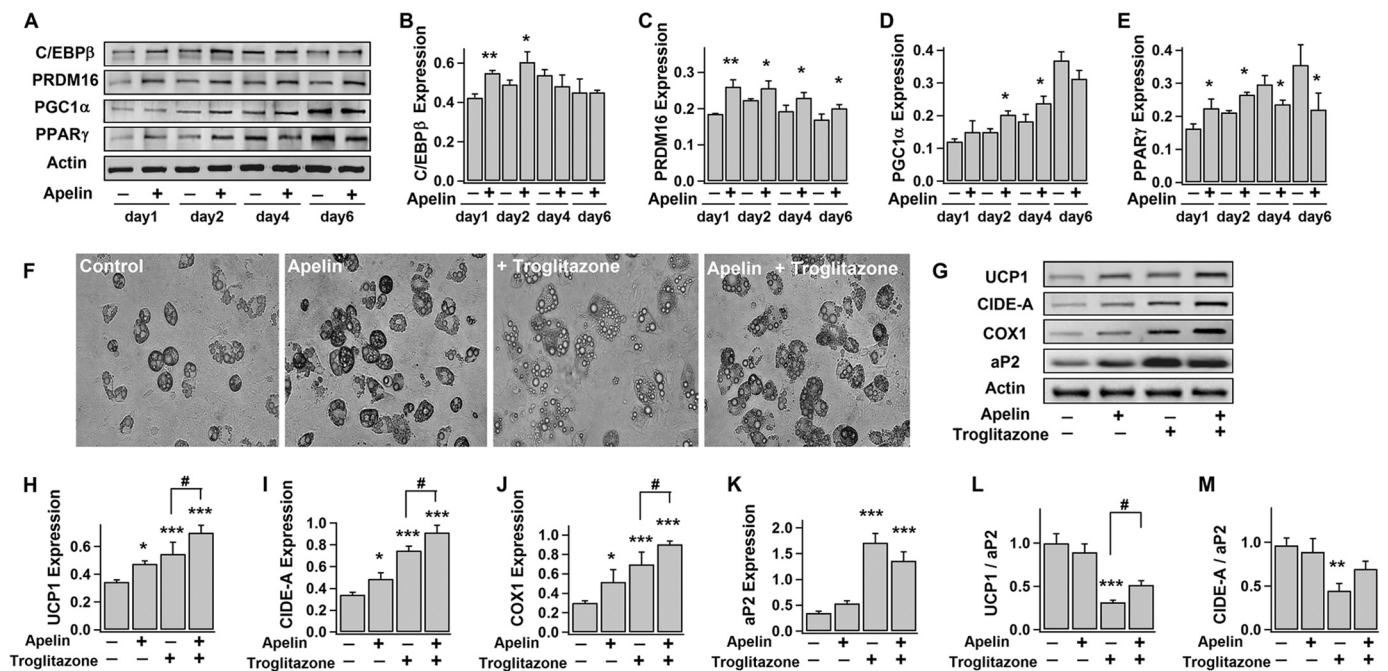


FIGURE 3. Apelin increases the expressions of brown adipogenic and thermogenic transcriptional factors. A–E, time course of the expressions of C/EBPβ (~45 kDa), PRDM16 (~170 kDa), PGC1α (~90 kDa), PPARγ (~57 kDa), and actin during brown adipocyte differentiation, without (–) or with (+) exposure to pyr-apelin13. The representative immunoblots and the statistics (mean ± S.E. (error bars), $n = 4$; blot densities were normalized to actin density; *, $p < 0.05$; **, $p < 0.01$ versus untreated; Student's t test) are shown in A and B–E, respectively. F–M, brown preadipocytes were induced to differentiate into brown adipocytes for 8–10 days, without (–) (control) or with (+) exposure to pyr-apelin13 and/or 10 μ M troglitazone (added at day 2). The representative bright field images of brown adipocytes and immunoblots of UCP1, CIDE-A, COX1, aP2, and actin expressions in brown adipocytes are shown in F and G, respectively. The statistics (mean ± S.E., $n = 4$) of the blot densities normalized to that of actin are shown in H–K. UCP1/aP2 and CIDE-A/aP2 are density ratios of UCP1, CIDE-A, and aP2 (L and M). Using one-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus untreated; #, $p < 0.05$ between the indicated pairs.

4, A and C), suggesting that apelin is only stimulatory to the basal metabolic activity.

Mitochondria with abundant UCP1 are responsible for metabolic activities in brown adipocytes. Mitochondrial biogenesis and morphological change (from tubular to spherical) accompany thermogenic activation in brown adipocytes (14, 57). We and others have shown that apelin increases mitochondrial biogenesis in white adipocytes and skeletal muscle cells (31, 34). As shown in Fig. 4G, tubular mitochondria are densely packed in brown adipocytes. Apelin treatment further increased its density, whereas mitochondria appeared shorter and more spherical in shape. TNF α secreted from macrophages in adipose tissue is a deterrent to brown thermogenesis (43, 44). In comparison, tubular mitochondria became more elongated in TNF α -treated brown adipocytes. Interestingly, in some brown adipocytes, mitochondria surround small lipid droplets, and these ring-shaped mitochondria are larger and more abundant in apelin-treated cells (data not shown). As shown by a previous study, mitochondrial rings are observed in brown adipocytes treated with the uncoupling proteins that are responsible for reduced membrane potential and increased oxygen consumption in mitochondria (58). We found that apelin treatment decreased the mitochondrial membrane potential (Fig. 4H) (as evidenced by the decreased fluorescence intensity of Mito-Tracker Red staining), without affecting cell viability (data not shown). Taken together, the evidence indicates that apelin stimulates the basal metabolic activities in brown adipocytes.

Apelin Increases the Browning Characteristics in White Adipocytes—In addition to classical brown adipocytes, certain white adipocytes can transform into brown-like adipocytes

(called beige cells) in response to various stimuli (e.g. cold exposure, etc.) (14). Beige cells are characterized by their multilocular lipid droplets, dark brown color due to high mitochondrial content, and expression of thermogenic and brown fat-specific genes (e.g. UCP1) (14). Because both apelin and APJ are abundantly expressed in white adipocytes (24, 59), we sought to investigate apelin's effects on browning of white adipocytes. Consistent with previous studies (13, 60), it was found that UCP1 expression in human white adipocytes is much lower than in brown adipocytes (Fig. 5). Similar to its stimulatory effect on brown adipocytes, apelin increased the expression of PRDM16, which is known to promote the browning of white adipocytes (14, 61) (Fig. 5, A and G). As expected, UCP1 and COX1 expressions were also significantly increased in apelin-treated human white (Fig. 5A) and 3T3-L1 adipocytes (mouse white preadipose cell line) (days 5–9) (Fig. 5G). Consistently, apelin treatment decreased the mitochondrial membrane potential and enhanced the basal activity of mitochondrial respiration (as evidenced by the increased OCR) (Fig. 5, C–E) in human white adipocytes. Some dark-colored multilocular brown adipocyte-like cells were also observed after apelin treatment (Fig. 5, B and F). All of these results indicate that apelin promotes the browning characteristics in white adipocytes.

We and others have previously reported that apelin release from white adipocytes increases over time during adipocyte differentiation (24). Using 3T3-L1 adipocytes, we examined whether apelin induces the brown-like phenotype at the late stage of adipocyte differentiation. As shown in Fig. 5G, apelin treatment (days 2–9) decreased the expression of aP2 (a marker protein for differentiated adipocytes) (62), suggesting that ape-

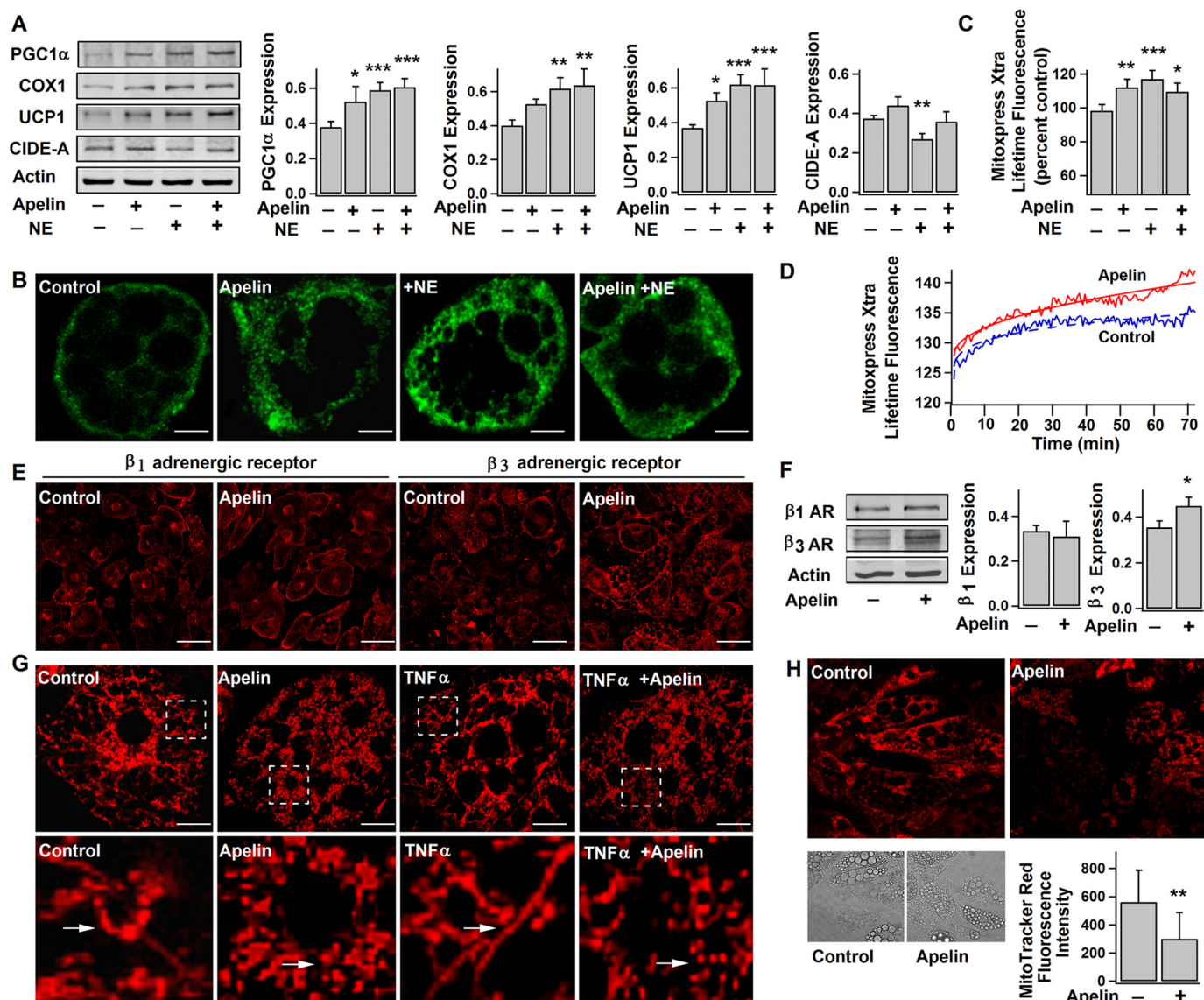


FIGURE 4. Apelin increases UCP1 expression and basal metabolic activity in brown adipocytes. After 3–4 days without (–) (control) or with (+) exposure to pyr-apelin 13, brown adipocytes (day 10) were treated without (–) or with (+) 1 μM NE for 6 h. *A*, representative immunoblots and statistics (mean ± S.E. (error bars), *n* = 4, blot densities were normalized to that of actin) of PGC1α, COX1, UCP1, CIDE-A, and actin expressions in brown adipocytes. Using one-way ANOVA, *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 versus untreated. *B*, confocal images of immunostained UCP1 in differently treated brown adipocytes. Scale bars, 10 μm. *C* and *D*, lifetime fluorescence value of MitoXpress-Xtra assessed as an indication of O₂ consumption rate in brown adipocytes. The statistics (mean ± S.E., *n* = 6 at 60 min) and the real-time responses of average fluorescence value are shown in *C* and *D*, respectively. *E*, confocal images of immunostained β1-AR and β3-AR in brown adipocytes. Scale bars, 50 μm. *F*, representative immunoblots of β1-AR (~65 kDa) and β3-AR (~44 kDa) expressions in brown adipocytes, and the statistics (mean ± S.E., *n* = 4) of the blot densities normalized to that of actin. *G*, the representative confocal images of mitochondria (detected by MitoTracker Red) (top lane) and their corresponding magnified images of insets (bottom lane) in brown adipocytes (day 10) after treatment without or with pyr-apelin 13 and/or 10 ng/ml TNFα (2–3 days). Scale bars, 10 μm. *H*, the representative confocal images showing the changes of mitochondrial membrane potential (detected by MitoTracker Red) and the statistics of fluorescence intensity (mean ± S.E., *n* = 12 cells, from 3–4 independent experiments). Using Student's *t* test, *, *p* < 0.05; **, *p* < 0.01 versus untreated.

lin hinders the terminal differentiation of white adipocytes. This is in line with the previous observation that apelin is able to decrease 3T3-L1 adipocyte differentiation (29). On the other hand, COX1, UCP1, and CIDE-A protein levels (brown adipocyte markers) were not reduced in apelin-treated cells (Fig. 5G). On the contrary, knockdown of apelin or APJ receptor decreased the expression ratios of UCP1/aP2 and CIDE-A/aP2 (data not shown). In summary, our results suggest that apelin apparently hinders maturation of white adipocytes while promoting the brown fat-like characteristics during the terminal phase of 3T3-L1 adipocyte differentiation.

We further examined apelin's effects on browning characteristics during the adipogenic differentiation of hMSCs. A recent study showed that, unless PRDM16 is overexpressed, hMSCs will only differentiate into white adipocytes with low UCP1 expression, even under ectopic expression of PPARγ (53). Similar to human white adipocytes, only low level of UCP1 expression was detected in adipocytes differentiated from hMSCs (Fig. 5H). In comparison, UCP1 expression was dramatically increased in apelin-treated cells, together with increased expression of PRDM16 (Fig. 5H), similar to its stimulatory effect on brown adipocytes. In contrast, leptin expression was

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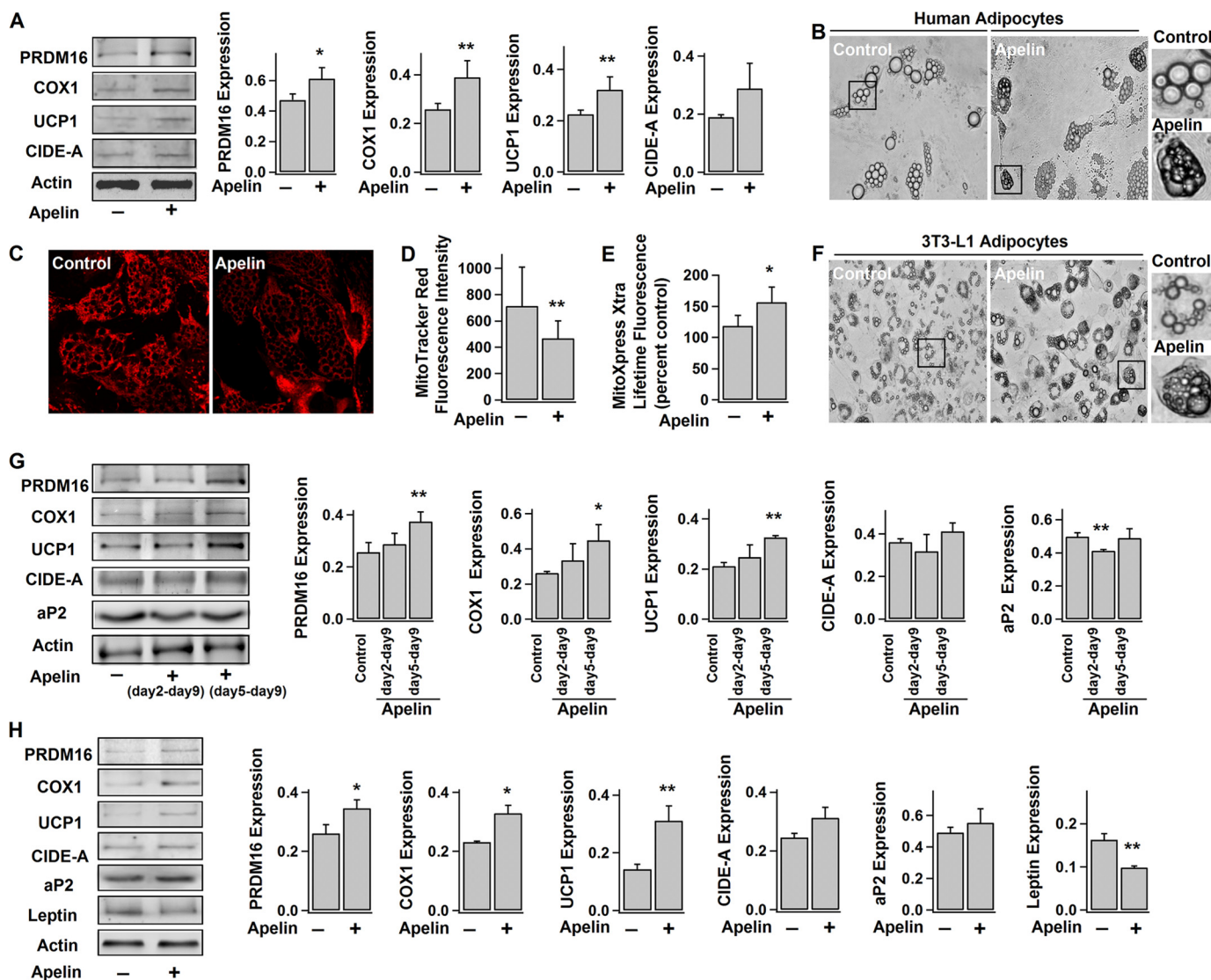


FIGURE 5. Apelin increases the browning characteristics in white adipocytes. A–E, human white adipocytes (day 14) were treated without (–) (control) or with (+) exposure to pyr-apelin13 for 4 days. F and G, 3T3-L1 adipocytes were treated without (–) (control) or with (+) exposure to pyr-apelin13 (days 2–9 or days 5–9). Shown are the representative immunoblots of protein expressions (PRDM16, COX1, UCP1, CIDE-A, aP2, and actin) in human (A) and 3T3-L1 adipocytes (G) with their respective statistics (mean \pm S.E. (error bars), $n = 4–5$, normalized to actin density). B and F, representative bright field images of differently treated human and 3T3-L1 adipocytes. C and D, representative confocal images of human adipocytes showing the changes of mitochondrial membrane potential (detected by MitoTracker Red) and the statistics of fluorescence intensity (mean \pm S.E., $n = 12$). E, lifetime fluorescence value of MitoXpress-Xtra assessed as an indication of OCR (mean \pm S.E., $n = 6$, at 60 min) in human adipocytes. H, human mesenchymal stem cells were induced to differentiate into adipocytes without (–) or with (+) exposure to pyr-apelin13. Shown are the representative immunoblots of PRDM16, COX1, UCP1, CIDE-A, aP2, leptin (~16 kDa), and actin expressions (day 14) and the statistics (mean \pm S.E., $n = 4–5$, normalized to actin density). Using Student's *t* test, *, $p < 0.05$; **, $p < 0.01$ versus control/no treatment.

significantly reduced. Previous studies suggest that leptin is white adipocyte-specific, because unilocular UCP1-negative adipocytes are leptin-positive, whereas multilocular UCP1-positive brown adipocytes are leptin-negative (63, 64). Our experiments on hMSCs further confirm that apelin is able to promote the browning characteristics in white adipocytes, at least in part, by stimulating the expression of PRDM16.

Apelin Promotes the Browning of WAT in Vivo—No significant difference in body weight (Fig. 6A) or food intake (data not shown) was observed in control (saline-treated) and apelin-treated mice. Interestingly, the apelin-treated mice exhibited significantly reduced weight of WAT (both inguinal and epididymal WAT), whereas the weight of interscapular BAT was similar to that of the control group (Fig. 6, B and C). Moreover, the

protein levels of PRDM16, COX1, and UCP1 (brown adipocyte markers) were elevated in WAT isolated from the apelin-treated mice (Fig. 6D).

To further confirm the browning effect of apelin, primary preadipocytes were isolated from mouse inguinal WAT and differentiated into white adipocytes. As shown in Fig. 6E, UCP1 (brown adipocyte-specific protein) level was much lower in these differentiated white adipocytes as compared with the brown adipocytes (differentiated from interscapular BAT preadipose cells from the same group of mice). In comparison, PRDM16, COX1, and UCP1 expressions were considerably up-regulated in apelin-treated white adipocytes (Fig. 6F), which is similar to the observations in human and 3T3-L1 white adipocytes (Fig. 5). Taken together, apelin's stimulation on WAT

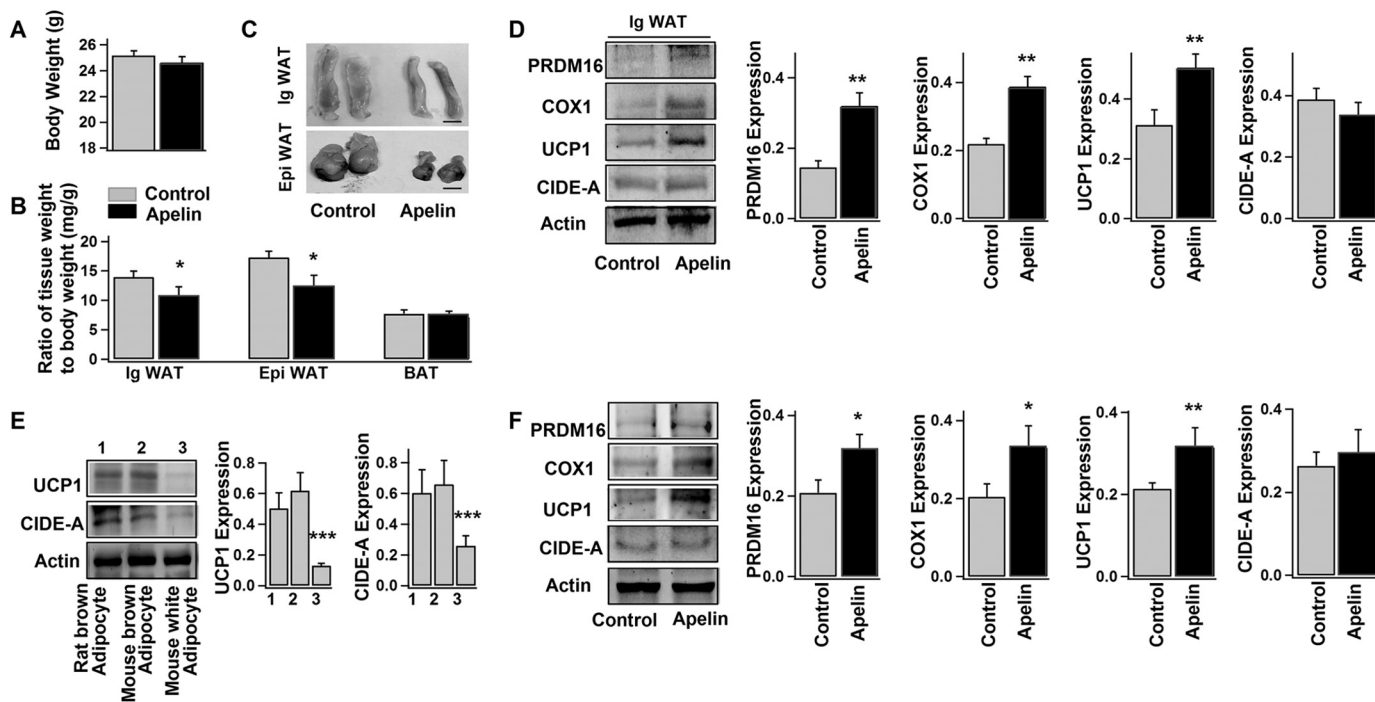


FIGURE 6. Both *in vivo* and *in vitro* mouse experiments confirm the apelin stimulation on WAT browning. Shown are average body weights (A) and weights of inguinal WAT (*Ig* WAT), epididymal WAT (*Epi* WAT), and interscapular BAT (B) relative to the total body weights of the control and apelin-treated mice (mean \pm S.E. (error bars), $n = 8$ mice/group). C, representative images of inguinal WAT and epididymal WAT isolated from the control and apelin-treated mice. Scale bar, 4 mm. D, representative immunoblots of protein expressions (PRDM16, COX1, UCP1, CIDE-A, and actin) in inguinal WAT isolated from the control and apelin-treated mice and the statistics of the blot density normalized to that of actin (mean \pm S.E., $n = 4$ mice/group). E, representative immunoblots and the statistics (mean \pm S.E., $n = 3$) of UCP1 and CIDE-A expressions in adipocytes (day 10) differentiated from different preadipose cells (1, preadipocytes isolated from rat interscapular BAT; 2, mouse interscapular BAT; 3, mouse inguinal WAT). F, representative immunoblots of protein expressions (PRDM16, COX1, UCP1, CIDE-A, and actin) and the respective statistics (mean \pm S.E., $n = 4$, normalized to actin density) from the white adipocytes (day 8, differentiated from preadipocytes of mouse inguinal WAT) treated without (-) (control) or with (+) pyr-apelin13 for 4 days. Using Student's *t* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control.

browning is confirmed by both *in vivo* and *in vitro* mouse experiments.

Apelin's Stimulatory Effects on Brown Adipogenesis and Browning of White Adipocytes Are Dependent on AMPK and PI3K/Akt Signaling Pathways—We and others have shown that apelin increases Akt and AMPK phosphorylation in white adipocytes (27, 28, 34). Here, we also found that apelin increased the activation (phosphorylation) of Akt and AMPK in primary rat brown preadipocytes (Fig. 7, A and B). As shown in Fig. 7 E and F, siRNA knockdown of Akt expression in brown preadipocytes precluded the brown adipogenesis, as evidenced by the decreased protein levels of PRDM16, COX1, UCP1, CIDE-A, and aP2. Apelin-induced brown adipogenesis was also effectively concealed in Akt-knock-out cells. In addition, AMPK protein knockdown in brown preadipocytes largely blocked the apelin-induced expressions of PRDM16, UCP1, CIDE-A, and aP2. Previous studies showed that both the PI3K/Akt and AMPK/mTOR signaling are important in brown adipogenesis (65, 66). Therefore, it may be concluded that apelin induces brown adipogenesis, at least in part, by activating the PI3K/Akt and AMPK signaling pathways.

Similar to the browning effects on human and mouse white adipocytes (Figs. 5A and 6F), apelin treatment (days 5–9) significantly increased the expressions of PRDM16, COX1, and UCP1 in 3T3-L1 white adipocytes (Fig. 7, G and H). Also, AMPK protein knockdown significantly blocked the apelin-induced expressions of PRDM16 and UCP1 (Fig. 7, G and H).

Apelin-induced UCP1 expression was also obscured in Akt-knock-out cells. This suggests that apelin's effect on browning of white adipocytes is also dependent on the AMPK signaling pathway.

Discussion

Adipose browning (including an increase of brown adipocyte differentiation in BAT and transformation of beige adipocytes in WAT) not only suppresses obesity through increased energy expenditure but also counteracts the detrimental effects of excess WAT (*e.g.* insulin resistance) (14, 15). An explosion of interest has been triggered because stimulating adipose browning may be a novel route to combat obesity and associated metabolic diseases. Increasing evidence is mounting to suggest that adipokines secreted by adipocytes play important roles in the self-modeling of adipose tissue (19, 29, 33). But their effects on browning are not well explored.

In this study, we present evidence that apelin secreted by both brown and white adipocytes stimulates adipose browning. Based on our experiments and previous studies, the following scenario may be proposed (Fig. 8). In obesity, adipose tissue is in chronic low grade inflammation, characterized by accumulation of macrophages and inflammatory cytokines, which contribute to the development of insulin resistance (45). Inflammatory factors (*e.g.* TNF α) released from macrophages suppress brown adipocyte formation (Fig. 2) (43, 44) and hence reduce BAT mass in obesity (3, 4). Consequently, decreased energy

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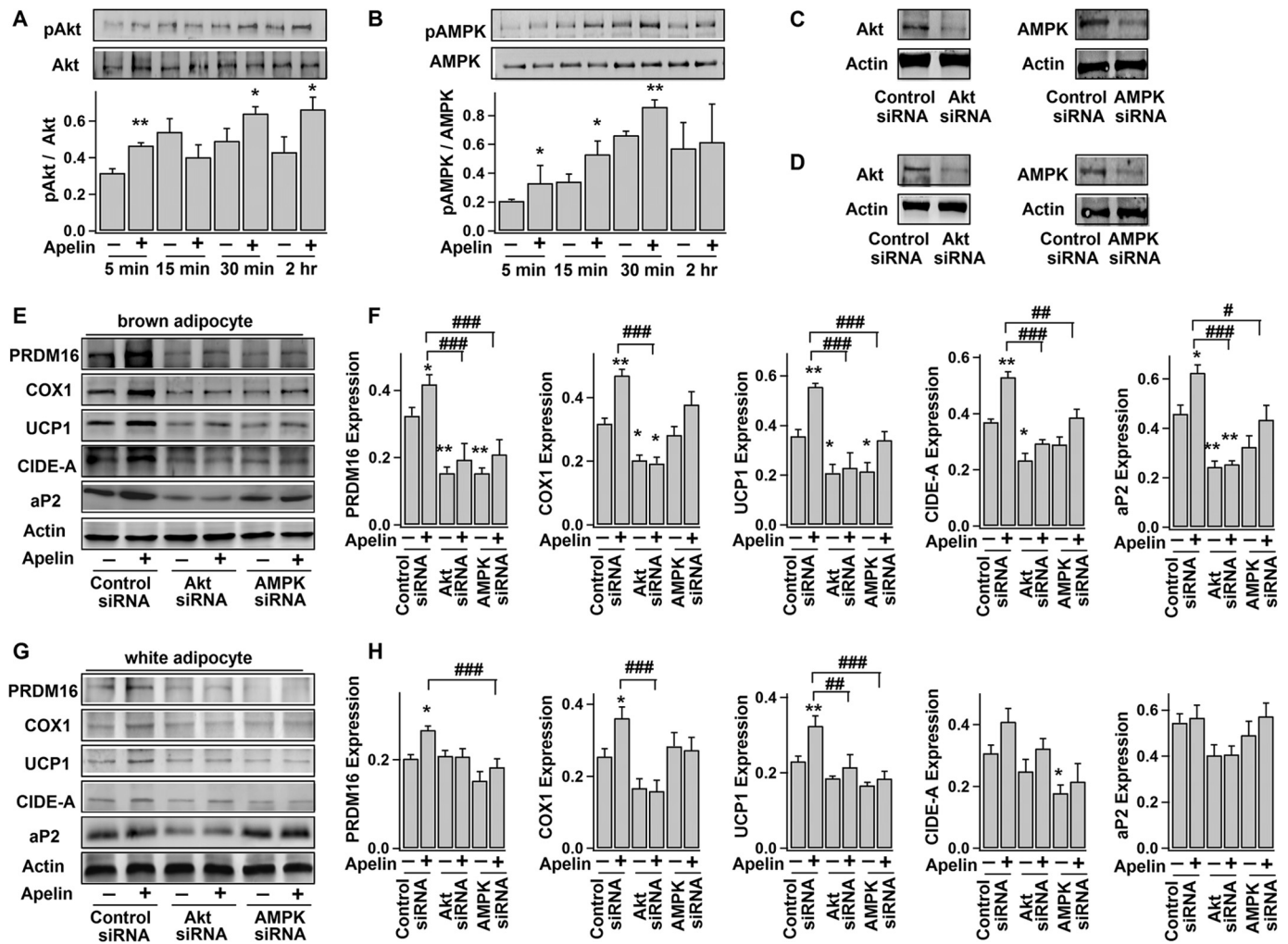


FIGURE 7. Signaling pathways underlying the apelin-induced brown adipocyte differentiation and browning characteristics of white adipocytes. *A* and *B*, time course of the phosphorylation and total protein levels of Akt (~60 kDa) and AMPK (~62 kDa) during brown adipocyte differentiation, without (–) (control) or with (+) exposure to pyr-apelin13. *Top panels*, representative immunoblots; *bottom panels*, statistics (mean ± S.E., $n = 3$) of the optical density ratio between pAkt and Akt or between pAMPK and AMPK (*, $p < 0.05$; **, $p < 0.01$ versus control; Student's *t* test). *C* and *D*, Western blot analyses of Akt and AMPK expressions in brown preadipocytes (*C*) and 3T3-L1 adipocytes (*D*), 2 days after transfection with control siRNA, Akt siRNA, or AMPK siRNA. *E* and *F*, brown preadipocytes were transfected with control siRNA, Akt siRNA, or AMPK siRNA 1–2 days before they were induced to differentiate into adipocytes, without (–) (control) or (+) with exposure to pyr-apelin13. Shown are representative immunoblots and the statistics (mean ± S.E., $n = 4$, normalized to actin density) of PRDM16, COX1, UCP1, CIDE-A, aP2, and actin expressions in differentiated brown adipocytes (day 10). *G* and *H*, 3T3-L1 adipocytes (day 4) were transfected with control siRNA, Akt siRNA, or AMPK siRNA, 1–2 days before treatment without (–) (control) or with (+) pyr-apelin13 (for 4 days). The representative immunoblots and the statistics (mean ± S.E., $n = 4$, normalized to actin density) are shown accordingly. Using one-way ANOVA, *, $p < 0.05$; **, $p < 0.01$ versus control; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ between the indicated pairs.

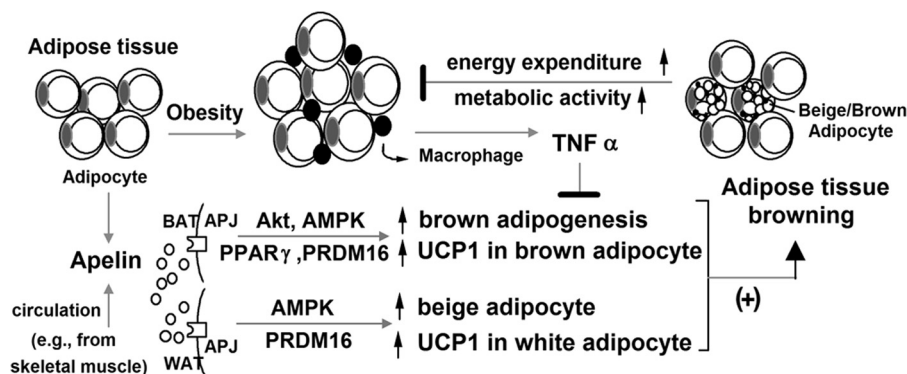


FIGURE 8. Apelin-induced adipose tissue browning.

consumption further aggravates the excess energy storage in WAT (1, 15). On the other hand, apelin expression in WAT and its plasma level are elevated in obesity (24). By interacting with APJ receptor, apelin activates PI3K/Akt and AMPK signaling in brown preadipocytes (Figs. 1 and 7), leading to increased expressions of brown adipogenic and thermogenic transcription factors (Fig. 3). As brown adipogenesis progresses, apelin expression increases in BAT, creating an autocrine positive feedback loop that enhances brown adipocyte differentiation (Fig. 1) and offsets the TNF α -impaired brown adipogenesis (Fig. 2). Apelin induces browning of white adipocytes too (Figs. 5 and 6). In addition to the increased number of brown or brown-like adipocytes, apelin also stimulates metabolic activity of adipocytes (Figs. 4 and 5). As a result, the body energy consumption increases (30), and WAT masses are reduced (Fig. 6). Moreover, apelin's inhibitory effect on white adipocyte differentiation offers effective negative feedback to hinder further expansion of WAT (29) (Fig. 5G). As described, the unique co-existence of apelin's positive and negative feedback loops compensate for the imbalanced development of BAT and WAT found in obesity. On the other hand, disruption of the apelin signaling by silencing apelin or APJ receptor expression decreases the number of brown adipocytes (Fig. 1) and increases the number of hypertrophied white adipocytes (29). The above proposed notion is consistent with the previous studies in rodents showing that 1) apelin treatment increases body temperature and O₂ consumption (30); 2) apelin treatment or BAT transplantation decreases body adiposity and insulin resistance (9, 25); and 3) apelin-knock-out or BAT-ablated mice exhibit increased central adiposity and insulin resistance (7, 28).

BAT metabolic activity (and thus energy consumption) can be enhanced by acute cold exposure (a few hours) (4, 67). This is due to increased sympathetic nervous system activity, whereby NE-induced cAMP accumulation promotes UCP1 expression and its uncoupling process (1, 14). Prolonged cold exposure (several days) is known to increase BAT mass as well as browning of WAT in humans (5, 68). Interestingly, apelin expression and release from white adipocytes are up-regulated by cAMP (59), and apelin plasma level is raised upon cold exposure (69). It is conceivable that cold-induced apelin increase is an adaptive reaction to stimulate adipose browning. NE-stimulated metabolic activities of brown adipocytes, however, cannot be further augmented by apelin (Fig. 4). This is attributable to the fact that apelin and NE antagonize each other on PKA/cAMP signaling. As shown previously, apelin inhibits cAMP production via the G_i protein-coupled APJ receptor (28, 70).

Exercise training also potentiates BAT thermogenesis and browning of WAT (15). Both exercise training and cold exposure increase the circulating levels of irisin and FGF21 (16, 17, 71). These recently found myokine and hepatokine stimulate brown fat-like thermogenesis in WAT (16, 17). Interestingly, in obese people, exercise only up-regulates muscle expression of apelin, without affecting irisin and FGF21 levels (72). A number of studies show that plasma apelin level is increased after exercise training and cold exposure (69, 73, 74). Hence, like irisin and FGF21, apelin could be regarded as an exercise- and cold-induced endocrine activator to promote adipose browning. We

have previously demonstrated in white adipocytes that apelin suppresses the release of fatty acids and reactive oxygen species (29, 34), which are key contributors to the pathogenesis of obesity-related metabolic disorders. Therefore, apelin is able to exert multiple beneficial effects through both white and brown adipocytes and may serve as a novel therapeutic target for obesity and related metabolic diseases.

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