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RESEARCH PAPER

L-Dihydroxyphenylalanine (L-Dopa) Induces Brown-like Phenotype in 3T3-L1 White Adipocytes via Activation of Dopaminergic and β3-adrenergic Receptors

Kiros Haddish and Jong Won Yun

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Abstract Due to its propensity to boost energy expenditure, browning of white fat is emerging as an intriguing and prospective target for therapeutic intervention in obesity. Here, we report that L-dihydroxyphenylalanine (L-Dopa), used as a gold standard therapy in Parkinson's disease, induces browning in 3T3-L1 adipocytes by increasing the expression levels of beige-specific marker genes such as *Cd137*, *Cited1*, *Cidea*, *Tbx1*, *Prdm16*, and *Ucp1*. In addition, exposure to L-Dopa induces a remarkable increase in the expressions of proteins involved in thermogenesis in white adipocytes. L-Dopa treatment also regulates 3T3-L1 adipocytes by markedly increasing protein expressions of p-AMPK, p-HSL, CPT1, ACOX1, and PPARα while decreasing FAS, ACC, C/EBPα, and PPARγ, suggesting enhanced lipolysis and fatty acid oxidation as well as reduced lipogenesis and adipogenesis, respectively. Molecular docking studies elucidated that L-Dopa binds to dopamine receptor D1 (DRD1) and β3-AR, thereby predicting the potential receptor candidates that activate protein kinase A (PKA), the master regulator of lipid metabolism. Mechanistic studies indicate that the browning potential of L-Dopa in 3T3-L1 white adipocytes is mediated by DRD1 and β3-AR activation, which consequently stimulates the PKA/p38 MAPK/ERK signaling pathway. In conclusion, L-Dopa appears to be a promising therapeutic candidate in the fight against obesity due to its inherent role in the browning of 3T3-L1 adipocytes via both the dopaminergic and adrenergic pathways. To our knowledge, this is the first report that demonstrates the browning potential of L-Dopa in white adipocytes. Our results may assist to expand the understanding on the contradictory findings in literature, related to the association between L-Dopa and weight loss observed in Parkinson's disease patients.

Keywords: anti-obesity, dopamine, L-Dopa, fat browning, 3T3-L1 cells

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1. Introduction

Obesity is the outcome due to an imbalance of caloric intake and energy expenditure [1]. Obesity is a substantial predisposing factor for complications arising in numerous common medical disorders including type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases [2]. Despite a wealth of scientific research and associated diverse mechanisms to effectively overcome the genetic factors and 'obesogenic environments', diminishing the rapid rise of obesity prevalence remains challenging [3]. This has necessitated the need for continuous efforts to fight obesity, and to reduce caloric intake while elevating energy expenditure, resulting in a decline in adipose tissue mass [4].

Adipose tissue is a highly dynamic and metabolically active organ that participates in several physiological processes, and is traditionally classified as white adipose tissue (WAT) and brown adipose tissue (BAT), which are responsible for energy storage and thermogenic energy expenditure, respectively [5]. Apart from the classical BAT, brown like phenotypes known as "beige" adipocytes could appear after thermogenic induction at anatomical sites

corresponding to WAT [6]. Both classical and beige adipocytes are positively associated with energy expenditure and negatively associated with adiposity in animal models and in humans [7]. In fact, uncoupling protein 1 (UCP1) is responsible for the specialized capacity of thermogenic respiration in both types of adipocytes [8]. This mitochondrial inner membrane carrier dissipates electrochemical forces leaked from the production of ATP and increases the rate of substrate oxidation to produce heat, which subsequently leads to weight loss [8].

Several pharmaceutical and bioactive compounds have recently been reported to encompass WAT browning capability as a strategy to increase energy expenditure, thereby alleviating weight gain [9-12]. These agents are capable of inducing WAT browning, which aids in combating the escalating occurrence of obesity by promoting the mitochondrial thermogenic capacity [13].

Levodopa (L-Dopa), also known as 3,4-dihydroxy-L-phenylalanine, is an amino acid derivative that is naturally produced in plants and animals, and is an immediate precursor of dopamine (DA) in the dopaminergic pathway [14]. Since the early 1960s, L-Dopa has continued to be the gold standard therapy for Parkinson's disease (PD) [15,16] due to the combination of a carbidopa inhibitor that irreversibly binds to pyridoxal 5′-phosphate, allowing L-Dopa to cross the blood-brain barrier more easily than DA [17].

Apart from PD, there are numerous reports indicating the efficacy of L-Dopa on the expressions of sex and growth hormones, and on cognitive functions [18-20]. The effect of L-Dopa as an anti-cancer agent as well as its role in the downregulation of NAFLD has also been documented [21]. Furthermore, intravenous administration of L-Dopa in animals and humans was reported to result in a rapid and significant increase in the levels of plasma glucose and plasma free fatty acids (FFA) [22]. Studies have also attempted to elucidate the association of L-Dopa with wight loss in PD patients. However, whether this weight loss is due to L-Dopa treatment or other factors, was inconsistently reported. According to one study, weight loss in 7 elderly PD patients treated with high doses of L-Dopa was due to the augmentation of the lipolytic actions of aging fat cells triggered by elevated levels of circulating insulin [23]. Contrarily, another study demonstrated that patients with advanced PD exhibit lower body mass index as compared to the control population obtained from an age-matched group, thereby suggesting that L-Dopa is directly responsible for weight loss in these PD patients [24]. Similarly, another independent study also reported that L-Dopa contributes to weight loss in PD patients [25,26]. These contradictory reports highlight the need for further research elucidating the relationship between L-Dopa and weight loss. The present study attempts to investigate whether L-Dopa, as part of the catecholamines, has a role in the browning of 3T3-L1 adipocytes to fight obesity.

2. Materials and Methods

2.1. Chemicals

L-Dopa with a purity of ≥ 98% was purchased from Sigma-Aldrich (St. Louis, MO, USA). KT-5720 was acquired from Cayman Chemical (Ann Arbor, MI, USA). SKF38393 was obtained from Abcam (Cambridge, UK). SCH 23390, BRL 37344, and L-748,337 were procured from Tocris Bioscience (Bristol, UK). All other chemicals used in this study were of analytical grade.

2.2. Cell culture and differentiation

3T3-L1 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Middlesex County, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 100 g/mL penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA) with passages between 7 and 11, at 37ºC in a 5% CO_2 incubator. A sufficient number of confluent cells were maintained in the differentiation induction medium containing 10 g/mL insulin (Sigma-Aldrich), 0.25 mM dexamethasone (Sigma-Aldrich), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) in DMEM, followed by culture in maturation medium supplemented with 10% FBS and 10 g/mL insulin in DMEM. The maturation media was replaced every two days. Unless otherwise stated, cells were maintained in complete media containing 10 μM L-Dopa or 10 μM DA for 6–8 days prior to analysis during treatments. Cytotoxicity of L-Dopa was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the manufacturer's instructions.

2.3. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

A total RNA isolation kit (RNA-spin; iNtRON Biotechnology, Seongnam, Korea) was used to isolate the total RNA from mature cells (4–8 days). The Maxime RT premix was used to synthesize cDNA from 1 μg of RNA (iNtRON Biotechnology). RT-PCR (Roche Lightcycler® 96; Roche Diagnostics, Basel, Switzerland) was used to quantitatively evaluate the transcription levels of genes using Power SYBR Green (Roche Diagnostics). PCR was performed in triplicate for each sample. All gene transcription levels were normalized to the level of β-actin. Table 1 shows the sequences of the primer sets employed in this study.

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

Gene	Forward	Reverse
DRD1	GGGGTTTTGGGAGAAGTGAC	AGTCACTTTTCGGGGATGCTG
Cited1	GGGGTAAAAGATCGCAAGGC	TGGTAGAAGGGGTGGCAGTA
Ppargc1α	ATGAATGCAGCGGTCTTAGC	AACAATGGCAGGGTTTGTTC
Prdm16	GATGGGAGATGCTGACGGAT	TGATCTGACACATGGCGAGG
Tbx1	AGCGAGGCGGAAGGGA	CCTGGTGACTGTGCTGAAGT
Tmem26	GAAACCAGTATTGCAGCACCC	CCAGACCGGTTCACATACCA
Ucp1	CCTGCCTCTCTCGGAAACAA	GTAGCGGGGTTTGATCCCAT
Cidea	CGGGAATAGCCAGAGTCACC	TGTGCATCGGATGTCGTAGG
Tfam	TAGGCACCGTATTGCGTGAG	GTGCTTTTAGCACGCTCCAC
Cox4	ACATTCAGGGTGCCTCTTTG	CATGGCAGAAGTGGGAGATT
Nrf1	GCTAATGGCCTGGTCCAGAT	CTGCGCTGTCCGATATCCTG

2.4. Oil Red O (ORO) staining

Preadipocytes of 3T3-L1 cells were seeded in a 6-well plate and allowed to reach 100% confluency. Cells were then treated with L-Dopa in differentiation and maturation media. After 72 h exposure, cells were washed with phosphate-buffered saline (PBS), fixed with 50% formalin for 1 h at room temperature, and washed again three times with deionized water. A mixture of ORO solution (0.6% ORO dye in isopropanol) and water (6:4 ratio) was layered onto the cells for 20 min, after which cells were washed four times with deionized water. The stained lipid droplets were subsequently visualized using an inverted microscope, and the intracellular lipid content was quantified from a standard expression of lipid droplets at 520 nm absorbance.

2.5. Immunoblot analysis

Radioimmunoprecipitation assay buffer (RIPA buffer) (Sigma-Aldrich) was applied to harvest the cell lysates, which were then homogenized and centrifuged at 13,000 × *g* for 30 min. The cell extract was diluted in 5X sample buffer (50 mM Tris at pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5%-mercaptoethanol, and 0.1% bromophenol blue), heated for 5 min at 95°C (polyacrylamide gel electrophoresis [PAGE]), and subsequently subjected to 8, 10, or 12% SDS-PAGE. Following electrophoresis, samples were transferred to a PVDF membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and blocked for 1 h with TBS-T (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% bovine serum albumin (BSA; Rocky Mountain Biologicals, Missoula, MT, USA), followed by overnight incubation with 1% BSA containing TBS-T and 1:1,000 dilution of various primary polyclonal antibodies including anti-actin, anti-DRD1, anti-ATGL, anti-ACC, anti-ATF2, anti-AMPK, anti-ACOX1, anti-β3-AR, anti-C/EBPα, anti-CPT1, anti-COX4, anti-ERK1/2, anti-FAS, anti-p-ACC, anti-PKA, anti-p38, anti-p-p38, anti-PPARγ, anti-p-AMPK, anti-p-ATF2, anti-CREB, anti-p-CREB, anti-ERK1/2, anti-p-ERK1/2, anti-PGC-1α, anti-UCP1, anti-CYT-C, and anti-PRDM16 (Abcam). Supplier and catalog numbers of each antibody are listed in Table S1. Probed membranes were washed twice, followed by 1 h incubation with horseradish peroxidase-conjugated anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG secondary antibody (1:1,000, Santa Cruz Biotechnology) in TBS-T buffer supplemented with 1% BSA. Detection was achieved through enhanced chemiluminescence with ImageQuant LAS500 (GE, Marlborough, MA, USA). Quantification of band intensities was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Immunofluorescence

For direct detection of UCP1 expression, 3T3-L1 cells were fixed with 96% methanol at 20°C, washed with PBS, followed by permeabilization with 0.25% Triton X-100 (Sigma-Aldrich), three washes with PBS, blocking with 1% BSA in PBS-T for 1 h, and overnight incubation at 4°C with anti-UCP1 antibody (Santa Cruz Biotechnology, 1:200 dilution). Probed cells were washed thrice with PBS and incubated for 4 h at room temperature with FITC-conjugated anti-goat, mouse secondary antibodies (1:400 dilution). Nuclei of the cells were simultaneously stained with DAPI (Invitrogen). An iRiS^{TM} digital cell imaging system (Logos Biosystems Inc., Seoul, Korea) was used to obtain the fluorescent images.

2.7. Molecular docking

Structure for the target protein dopamine receptor D1 (DRD1) with ID: 7JVQ was obtained from PDBe (https://www.ebi.ac.uk/pdbe/). Structure for beta-3 adrenergic receptor (β3-AR) with ID: 2CDW was retrieved from the protein data bank (https://www.rcsb.org/) and validated with protein database UniProt (https://www.uniprot.org/). The

target protein structure was optimized prior to initiation of the docking, by first removing the water molecules, ligand atoms and unwanted heteroatoms using Auto Dock, v4.2.6. Next, the hydrogen atoms and gasterier charges were added. The three- dimensional structure of L-Dopa was retrieved from DrugBank Online (https://go.drugbank.com). AutoDock 4.2.6 was used for molecular docking performance and analysis.

2.8. Statistical analysis

All data are presented as the mean ± standard deviation, and at least three independent experiments were performed for each data. The Statistical Package of Social Science (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) tool was used to examine statistical significance among several groups. A Student's *t*-test was used to assess data in circumstances when there were only two groups. Tukey's post-hoc test was used to evaluate differences between more than three experimental groups using the one-way or two-way analysis of variance (ANOVA). Statistical significance is denoted as either $p < 0.05$ or $p < 0.01$.

3. Results

3.1. L-Dopa enhances brown- and beige fat-specific markers in 3T3-L1 white adipocytes

MTT assay was first performed to determine cytotoxicity of L-Dopa, with DA as a comparison target, in 3T3-L1 preadipocytes. There was no significant influence on the cell viability of 3T3-L1 white adipocytes after exposure to DA and L-Dopa doses up to 10 μM (Figs. 1A and 1B, respectively). In addition, both DA and L-Dopa treatment at 10 μM significantly increased the expressions of beige-fat marker genes such as *Cd137*, C*idea*, *Cited1*, and *Tbx1*, as well as the core set of brown-fat marker genes including *Ppargc1a*, *Prdm16*, and *Ucp1* (Figs. 1C and 1D). Furthermore, as presented in Figs. 1A and 1B, treatment with DA and L-Dopa dose-dependently increased the protein expression levels of brown fat-specific markers (UCP1, PGC-1α, and PR domain containing 16 [PRDM16]).

3.2. L-Dopa promotes lipid metabolism in 3T3-L1 white adipocytes

Next, we examined whether L-Dopa affects the lipid metabolism in 3T3-L1 white adipocytes. To achieve this, we first determined the expression level of acetyl-CoA carboxylase (ACC; a rate-limiting enzyme in lipogenesis), which was lowered after L-Dopa treatment, with a concurrent increase in the ratio of p-ACC to total ACC mediated by AMP-activated protein kinase (AMPK) activation. Fatty acid synthase (FAS) was also downregulated subsequent to L-Dopa treatment of 3T3-L1 white adipocytes (Fig. 2A). Before and after L-Dopa treatment, the expression levels of lipolysis-related proteins, such as phosphorylated hormone-sensitive lipase (p-HSL) and adipocyte triglyceride lipase (ATGL), were also examined. L-Dopa enhanced lipolysis by elevating the expressions of p-HSL and ATGL (Fig. 2B). L-Dopa treatment also resulted in a significant increase in the expression levels of acyl-coenzyme A oxidase 1 (ACOX1), carnitine palmitoyltransferase 1 (CPT1), and peroxisome proliferator-activated receptor α (PPARα), implying an increase in the fat-oxidative capability (Fig. 2B). The levels of protein expressions of essential adipogenic transcriptional factors (C/EBP and PPARγ) were also measured, and were determined to be reduced as a result of L-Dopa treatment, implying a reduction in adipogenesis (Fig. 2C). Decrease in the number of fat droplets after treatment with L-Dopa in 3T3-L1 cells was further confirmed by ORO staining (Fig. 2D).

3.3. L-Dopa increases mitochondrial biogenesis in 3T3-L1 white adipocytes

Treatment of 3T3-L1 cells with L-Dopa significantly raised the expression levels of mitochondrial genes (*Cox4*, *Nrf1*, and *Tfam*) (Fig. 2E), as well as mitochondrial biogenic proteins (CYT-C, COX4) (Fig. 2E). Furthermore, staining of differentiated adipocytes with MitoTracker™ Red revealed stronger signals in the L-Dopa-treated adipocytes than in control cells, thereby further validating the effect of L-Dopa (Fig. 2F).

3.4. L-Dopa interacts with DRD1 and β3-AR

To elucidate the receptors responsible for the activity of L-Dopa in white adipocytes, we targeted DRD1 and β3-AR and predicted the binding activity of L-Dopa with both the receptors using molecular docking techniques. We observed that L-Dopa binds with DRD1 with a minimum binding energy of -19.79 kJ/mol (Table S2) and 1H bond at residue Phe 341 and other binding residues Pro 328, Ile 329, Ala 332, and Gln 338 (Fig. 3B). Interestingly, L-Dopa also showed binding activity with β3-AR with binding energy of -17.57 kJ/mol (Table S2) at residues Cys 129, Ser 209, Ser 210, and Phe 213 (Fig. 3A). The predicted binding activities of L-Dopa with DRD1 and β3-AR allowed us to detect the target receptors required for further *in vitro* mechanistic studies undertaken to identify the browning signaling pathway activated by L-Dopa in white adipocytes.

3.5. L-Dopa induces browning of white adipocytes through DRD1 and β3-AR activation

The day-dependent mRNA expressions of DRD1 in 3T3-

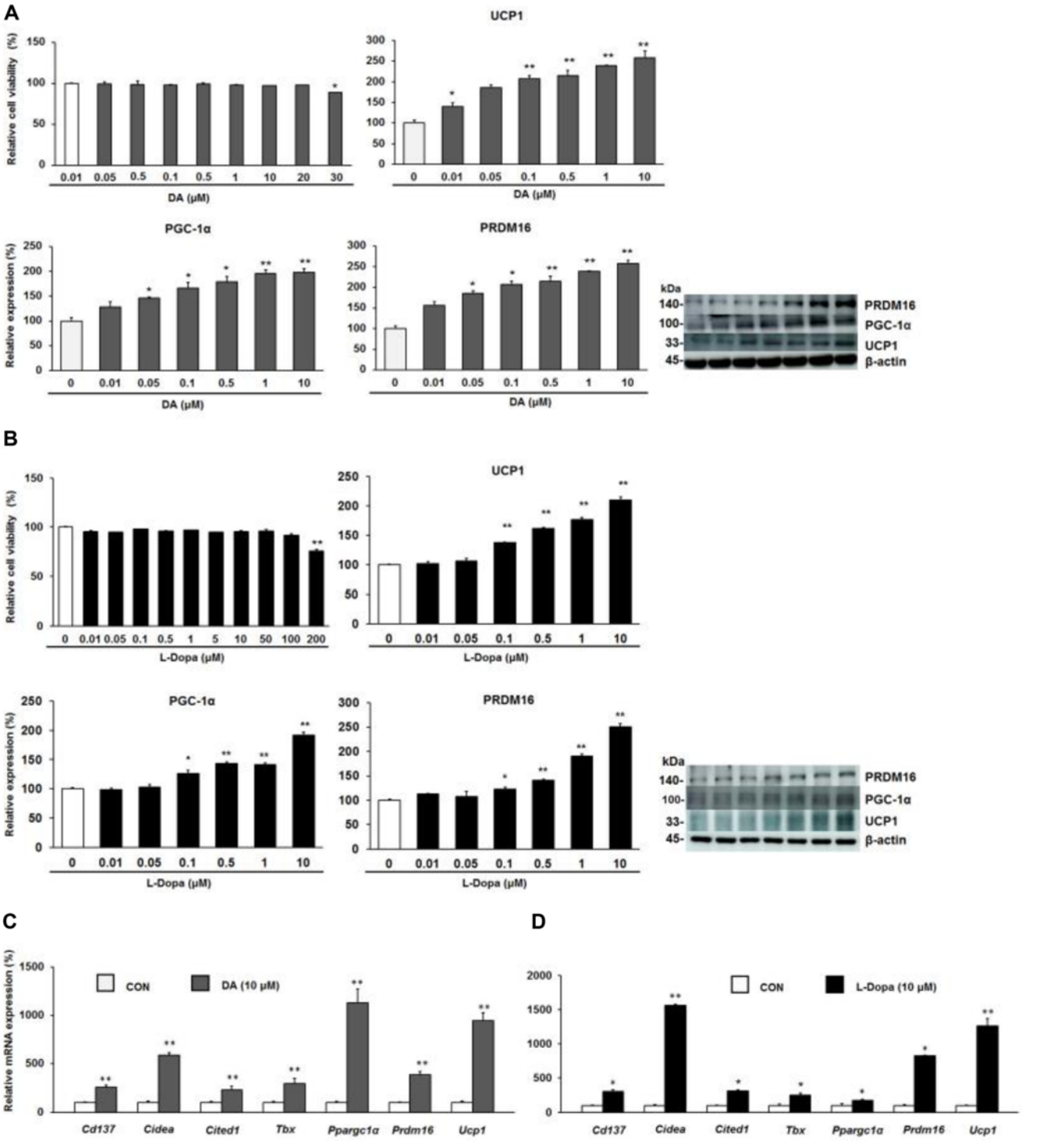

Fig. 1. Cytotoxicity of dopamine (DA) and levodopa (L-Dopa) in 3T3-L1 cells, as assessed by MTT assay. Both DA (A) and L-Dopa (B) induce browning of white adipocytes that gives rise to the activation of essential brown adipocyte marker proteins. Both DA (C) and L-Dopa (D) activate the expressions of beige fat-specific genes. Histograms display triple independent experiments for immunoblot analysis, and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and L-Dopa-treated 3T3-L1 cells is indicated by *$p < 0.05$ or **$p < 0.01$. Statistical significance between control, DA and L-Dopa-treated 3T3-L1 cells is shown by *$p < 0.05$ or **$p < 0.01$. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ANOVA: analysis of variance, UCP1: uncoupling protein 1, PGC-1α: peroxisome proliferator-activated receptor gamma co-activator 1-alpha, PRDM16: PR domain containing 16.

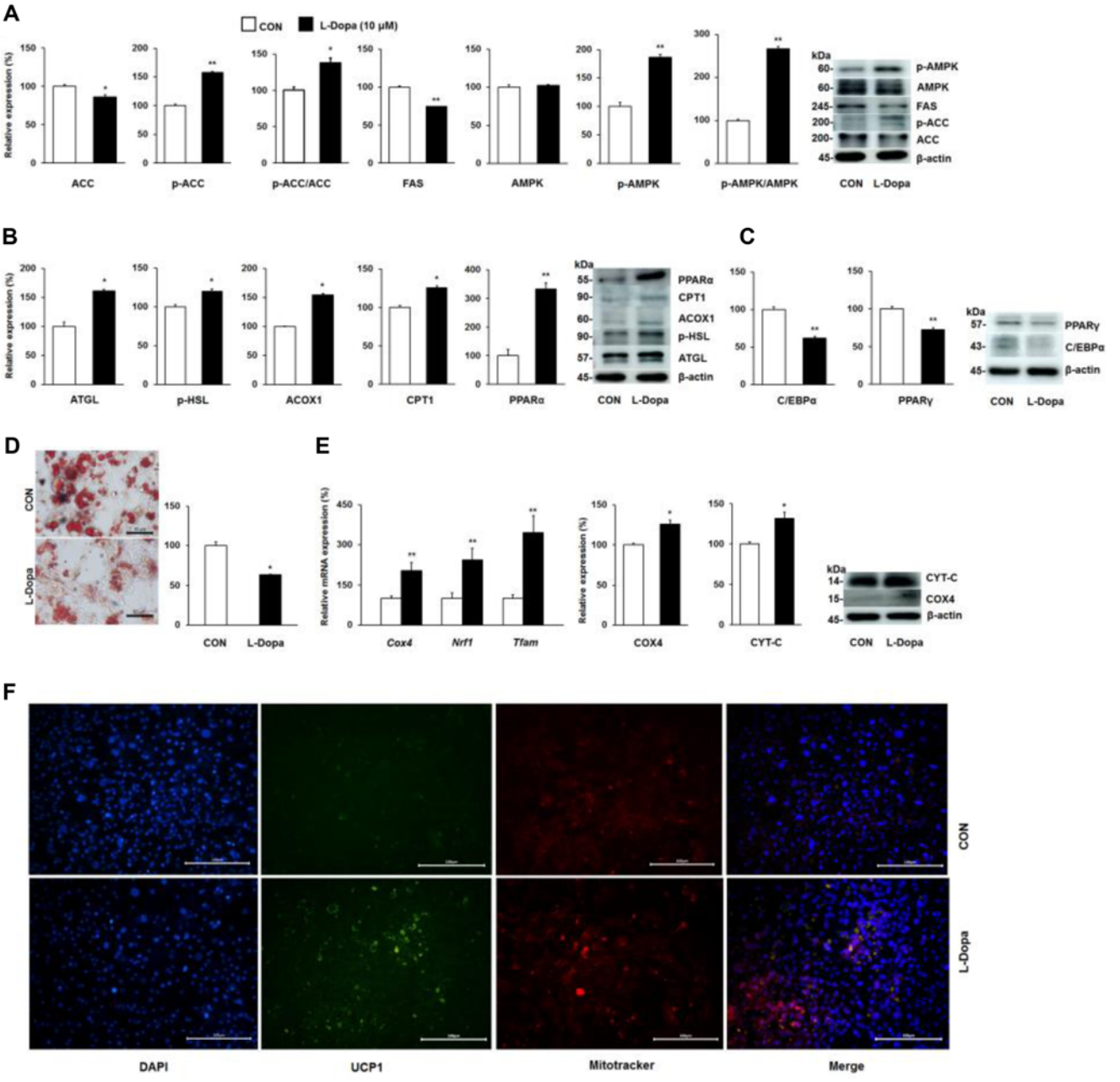

Fig. 2. Levodopa (L-Dopa) inhibits lipogenic marker proteins in 3T3-L1 white adipocyte cells (A), but enhances the expressions of lipolytic and fat oxidation protein markers (B). L-Dopa also reduces expression of adipogenic proteins in white adipocytes (C) and fat accumulation, as estimated by Oil Red O staining (D). L-Dopa upregulates the expressions of genes and proteins (E) responsible for mitochondrial biogenesis in 3T3-L1 cells. Immunohistochemical staining shows increased expression of uncoupling protein 1 (UCP1) upon L-Dopa exposure (F) (×40 magnification; scale bar = 100 μm). Histograms display triple independent experiments for immunoblot analysis, and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and L-Dopa treated 3T3-L1 cells is indicated by $*p < 0.05$ or $**p < 0.01$. Statistical significance between control, and DA- and L-DA treated 3T3-L1 cells is shown by $*p < 0.05$ or $**p < 0.01$. ANOVA: analysis of variance, ACC: acyl-CoA carboxylase, FAS: fatty acid synthase, AMPK: AMP-activated protein kinase, ATGL: adipocyte triglyceride lipase, p-HSL: phosphorylated hormone-sensitive lipase, ACOX1: acyl-coenzyme A oxidase 1, CPT1: carnitine palmitoyltransferase 1, PPARα: peroxisome proliferator-activated receptor α, COX4: cyclooxygenase-4, CYT-C: cytochrome C.

L1 cells were evaluated, and the receptors are expressed linearly until the completion of differentiation, after which they slowly decline during the maturation stage (Fig. 4A). Subsequently, we examined the expression levels of both

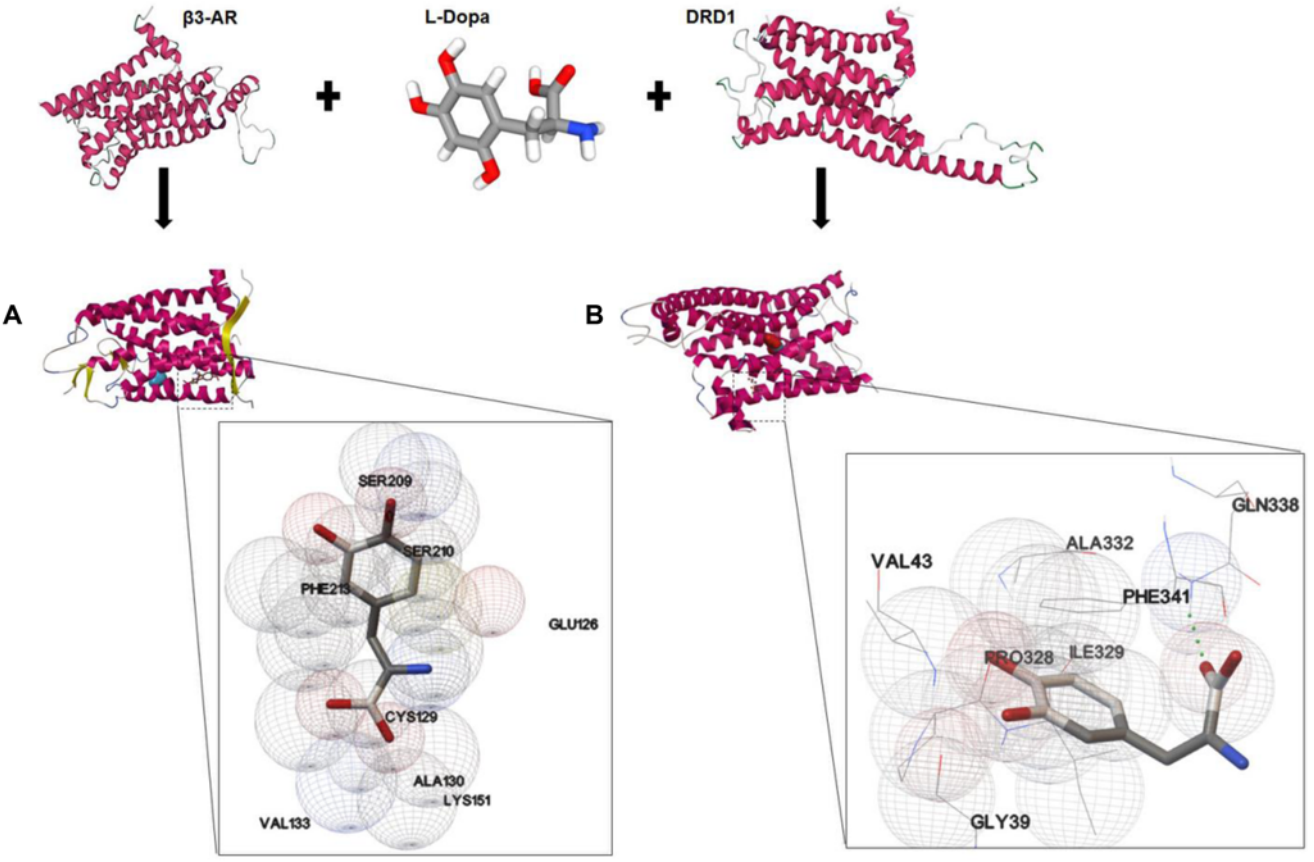

Fig. 3. Levodopa (L-Dopa) interacts with dopamine receptor D1 (DRD1) and beta-3 adrenergic receptor (β3-AR). Three-dimensional structure of L-Dopa, docked DRD1 to form strong binding complex with 1H bond (B). L-Dopa binds with β3-AR to form the docked complex with weak forces (A). All data represented by AutoDock generated interpretations.

receptors in 3T3-L1 white adipocytes treated with L-Dopa. As presented in Fig. 4B, L-Dopa increases DRD1 level in white adipocyte cells. We next investigated the molecular mechanisms underlying the browning activity of L-Dopa, for which we determined the expression levels of various possible signaling molecules linked to fat browning. As shown in Fig. 4B, β3-AR, DRD1, and protein kinase A (PKA) are positively regulated in L-Dopa-treated 3T3-L1 cells. L-Dopa also upregulated the expressions of downstream pathway molecules particularly p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), cAMP response element-binding protein (CREB), and activating transcription factor 2 (ATF2). L-Dopa further activated the phosphorylated form of these pathway molecules *viz*. p-p38, p-ERK1/2, p-CREB, and p-ATF2. The ratio of the total protein of these pathways were increased to +205.6% for p-p38/p38 MAPK, +127.27% for p-ERK1/2/ERK1/2, +121.5% for p-CREB/CREB, and +127.3% for p-ATF2/ATF2. To further understand the molecular mechanisms behind the browning impact of L-Dopa, the cells were treated with DRD1 agonist and antagonist (SKF38393 and SCH 23390, respectively), β3-AR agonist and antagonist (BRL 37344 and L-748,337, respectively) and PKA antagonist (KT5720), with or without L-Dopa (10 μM) during 7 days of differentiation. Subsequently, the expression levels of proteins that are expected to be the downstream target molecules of DRD1 and β3-AR (PKA, p38 MAPK, ERK1/2, CREB, and ATF2) as well as BAT-specific proteins (UCP1, peroxisome proliferator-activated receptor gamma co-activator 1-alpha [PGC-1α], and PRDM16) were examined. Our results demonstrate that the antagonists significantly suppress both DRD1 and β3-AR, thereby resulting in lower expressions of PKA, p38 MAPK and p-p38, ERK1/2 and p-ERK1/2, ATF2 and p-ATF2 as well as UCP1, PGC-1α and PRDM16, whereas the synergistic treatment of L-Dopa and the agonists elevated the expression levels of the proteins (Figs. 5 and 6). The antagonists reversed the agonistic action of L-Dopa, resulting in lower expressions of DRD1 and β3-AR along with their possible downstream targets (PKA, p38 MAPK and p-p38, ERK1/2 and p-ERK1/2, ATF2 and p-ATF2) as well as browning markers (Figs. 5 and 6), thereby

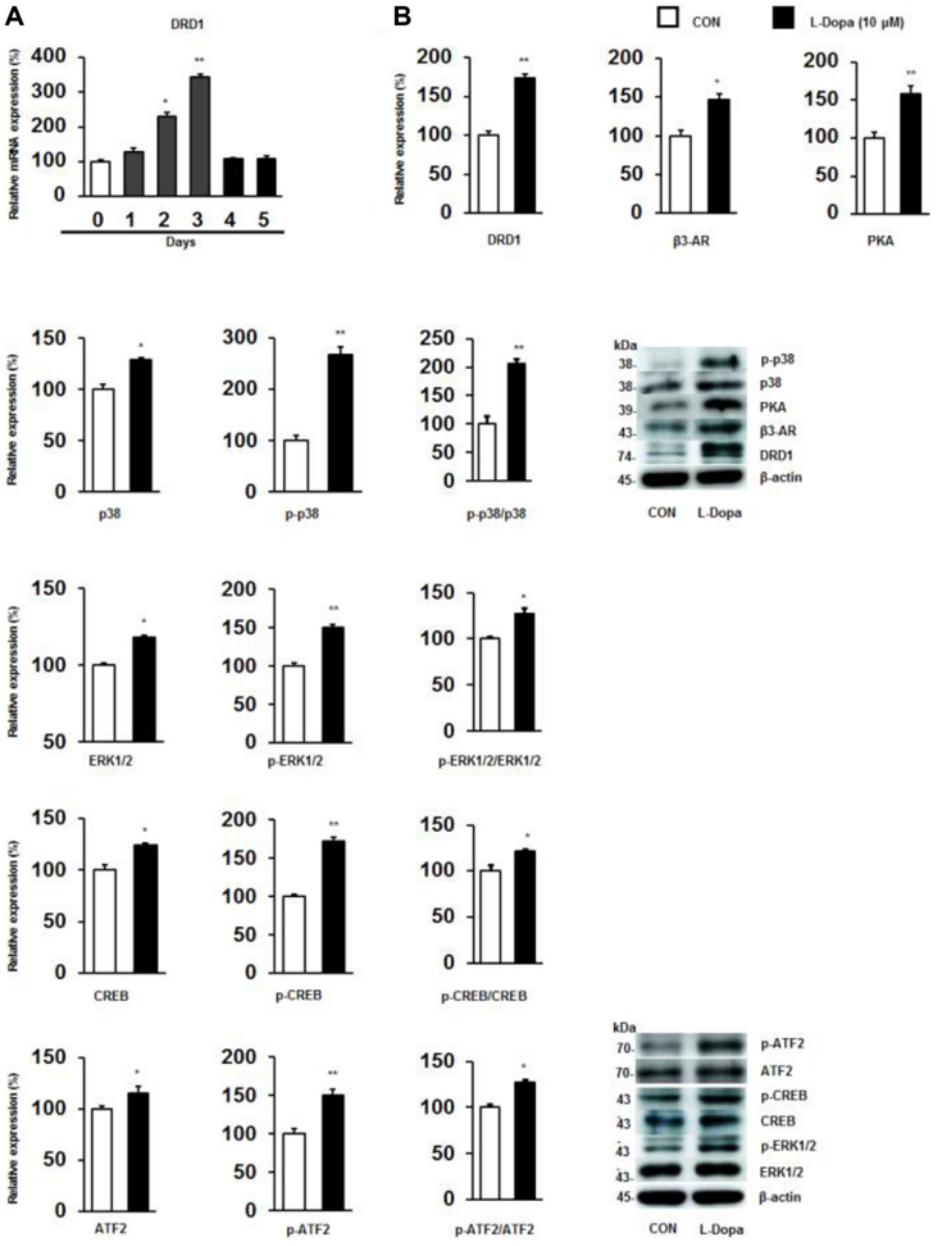

Fig. 4. Day-dependent mRNA expression of dopamine receptor D1 (DRD1) in 3T3-L1 cells exhibits linear expression of the receptors up to the end of differentiation, and a slow decline after the maturation stage (A). Levodopa (L-Dopa) activates DRD1, beta-3 adrenergic receptor (β3-AR), protein kinase A (PKA), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), CREB, and activating transcription factor 2 (ATF2) (B). Histograms show triple independent experiments for immunoblotting analysis and polymerase chain reaction analysis and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and L-Dopa treated 3T3-L1 cells is indicated by *$p < 0.05$ or **$p < 0.01$. ANOVA: analysis of variance.

demonstrating that DRD1 plays a critical role in the browning of white fat. Additional mechanistic study using PKA antagonist (KT-5720) supported our hypothesis that the browning effect of L-Dopa is evidently regulated by PKA and its downstream targets p38 MAPK/ERK/ATF2, with increasing expressions of thermogenic proteins (Fig. 7). Based on our overall findings, Fig. 8 depicts a schematic representation of the multiple paths for the involvement of L-Dopa in 3T3-L1 white adipocyte browning events.

4. Discussion

The most broadly studied biological application of L-Dopa

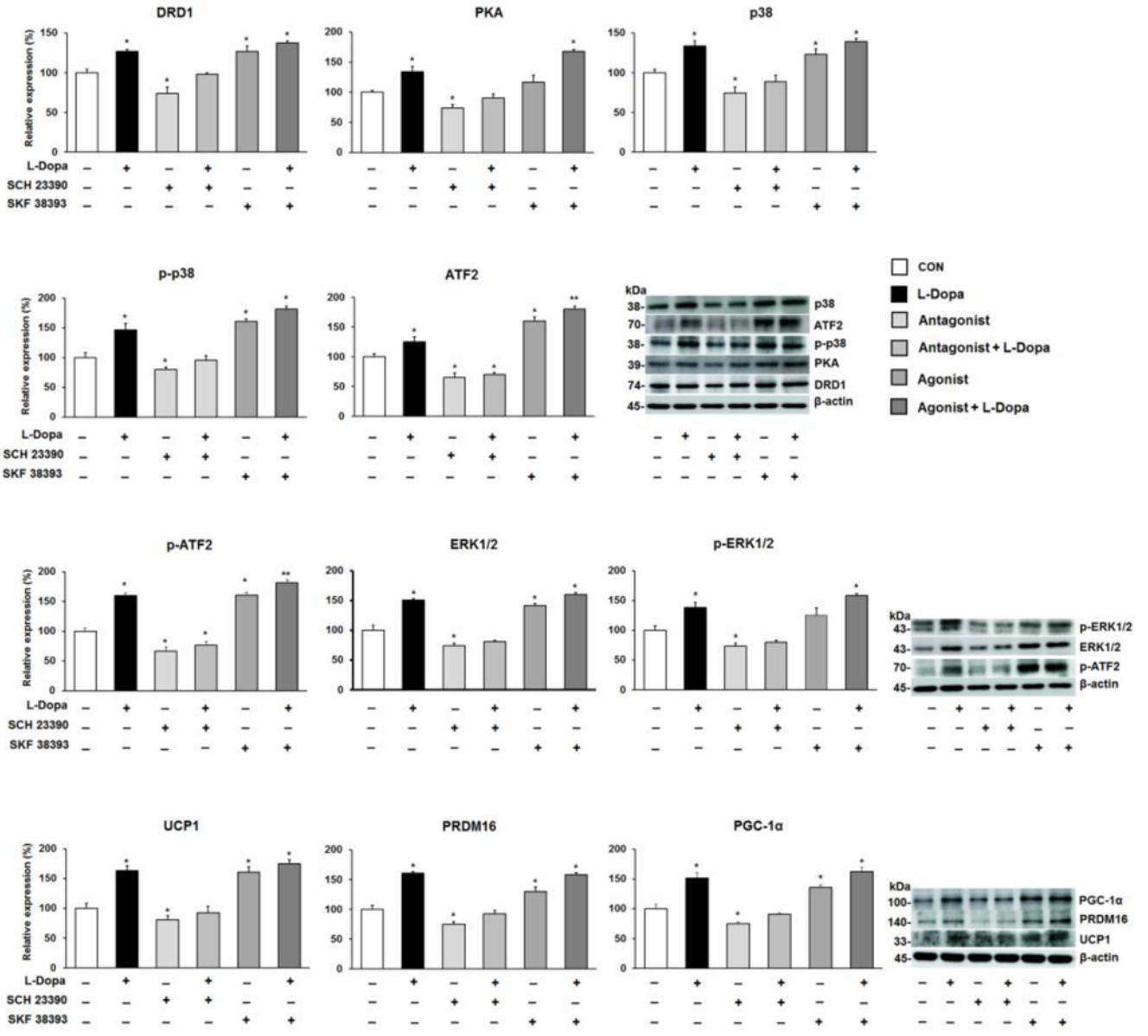

Fig. 5. Effect of levodopa (L-Dopa) on the expressions of fat browning-associated signaling molecules. L-Dopa stimulates dopamine receptor D1 (DRD1), thereby activating the expressions of protein kinase A (PKA), p38 mitogen-activated protein kinase (p38 MAPK), and extracellular signal-regulated kinase 1/2 (ERK1/2) and activating transcription factor 2 (ATF2), as well as increasing the expressions of browning markers uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) and PR domain containing 16 (PRDM16). Histograms show triple independent experiments for immunoblotting analysis and polymerase chain reaction analysis, and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and L-Dopa treated 3T3-L1 cells is indicated by *$p < 0.05$ or **$p < 0.01$. ANOVA: analysis of variance.

is its role as the DA replacement agent for the treatment of PD. Currently, this is the gold standard therapy for PD [15,16]. A body of literature with contradictory data illustrates that the incidence of underweight PD patients receiving long-term L-Dopa medication is attributable to this therapy [23-28]. However, in all these conflicting spectra of scientific reports, the exact molecular mechanisms associated with energy metabolism and browning capability of L-Dopa remains unclear.

In this study, we explored the possible browning potential of L-Dopa, a direct precursor of DA. In the first approach, we established the modulatory roles of DA in inducing the brown adipocyte-like phenotype in 3T3-L1 adipocytes, based on augmented expressions of beige-specific and

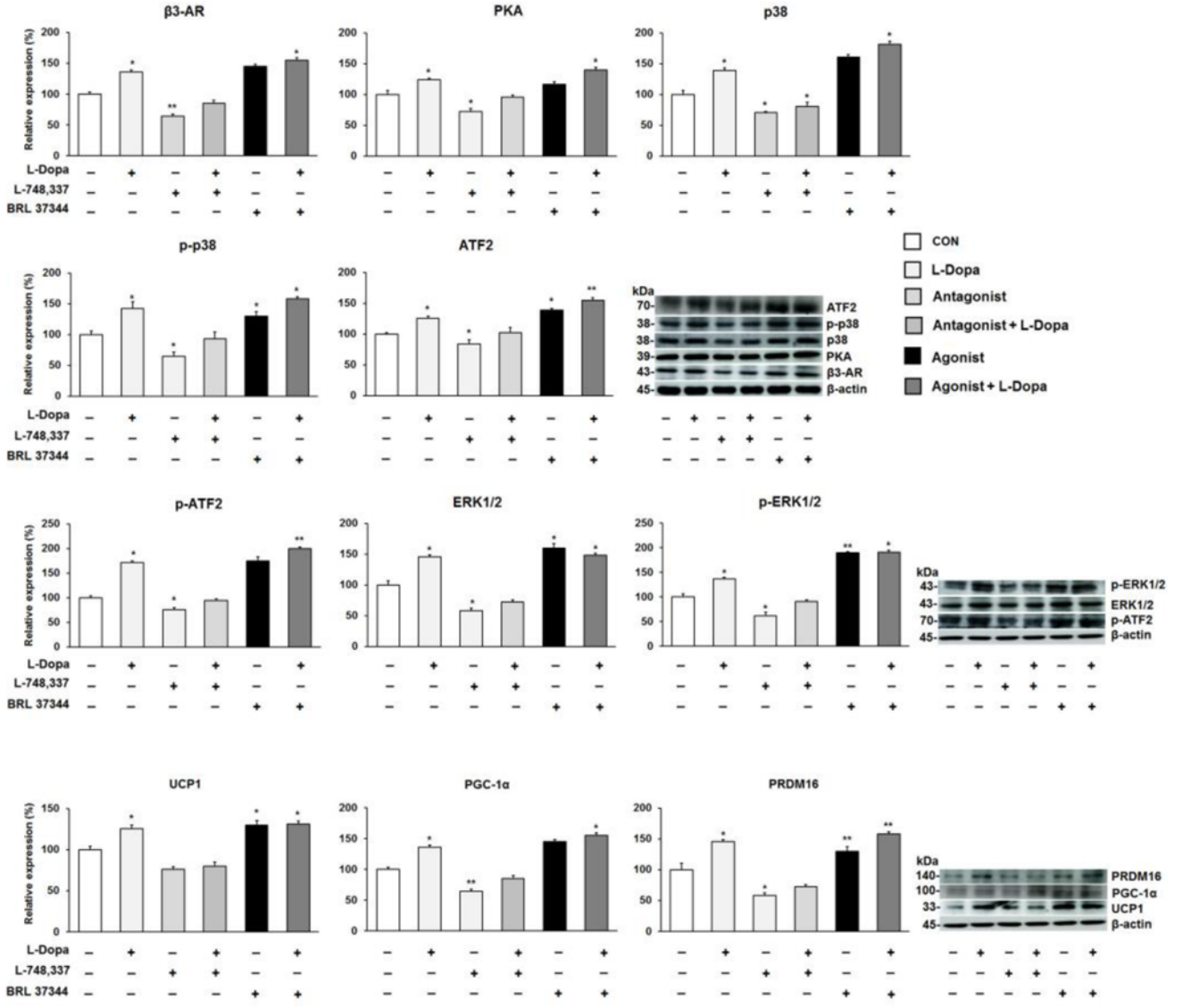

Fig. 6. Levodopa (L-Dopa) simulates browning of 3T3-L1 adipocytes via activation of beta-3 adrenergic receptor (β3-AR). Impairment of β3-AR by the antagonist results in reduced expressions of its downstream targets and browning markers. Treatment with the β3-AR agonist in combination with L-Dopa reverses the process, with increase in the expression levels of the target molecules and browning marker proteins. Histograms show triple independent experiments for immunoblotting analysis and polymerase chain reaction analysis, and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and L-Dopa treated 3T3-L1 cells is indicated by *$p < 0.05$ or **$p < 0.01$. ANOVA: analysis of variance, PKA: protein kinase A, p38 MAPK: p38 mitogen-activated protein kinase, ATF2: activating transcription factor 2, ERK1/2: extracellular signal-regulated kinase 1/2, UCP1: uncoupling protein 1, PGC-1α: peroxisome proliferator-activated receptor gamma co-activator 1-alpha, PRDM16: PR domain containing 16.

thermogenic markers. This finding is consistent with a previous study that demonstrates the direct stimulatory effects of DA on mitochondrial thermogenesis and mass in brown adipocytes, resulting in the activation of browning key protein markers [29]. Our findings for the immunofluorescence study also reveal stronger signals of UCP1 in the L-Dopa-treated adipocytes thereby further validating the effect of L-Dopa on the expression and localization of UCP1. The brown-fat marker proteins and genes (*Cd137*, *Cited1*, *Cidea*, *Tbx1*, *Ppargc-1α*, *Prdm16*, and *Ucp1*) are reported to be important in regulating the fate, development, and function of adipocyte precursor cell lineages as well as brown adipocytes [30]. Earlier reports have shown the distinct molecular signatures in the induction of brown adipocyte-like cells, which are also prevalent in beige adipocytes [31]. PRDM16 and PGC-1α are also well-

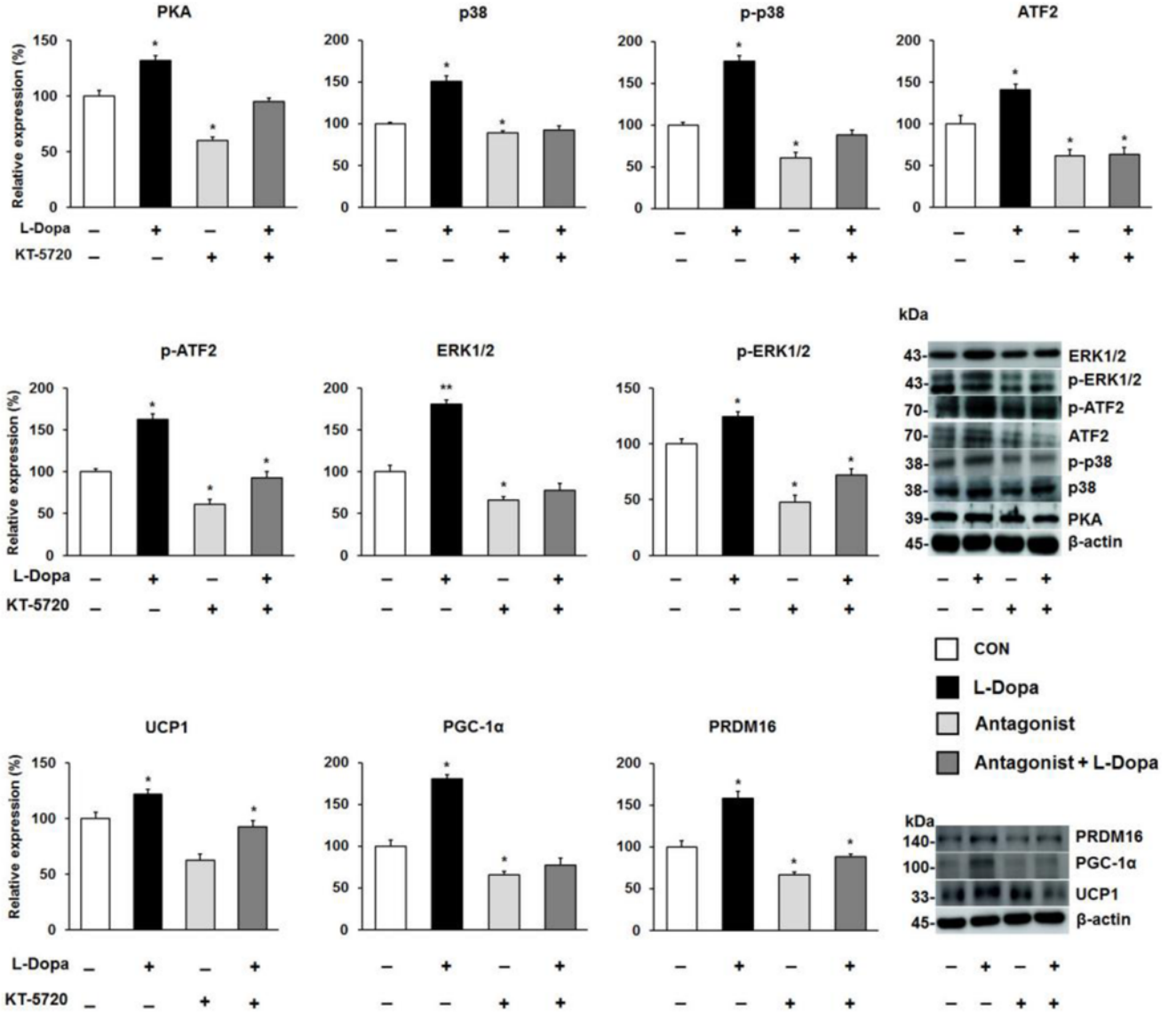

Fig. 7. Levodopa (L-Dopa) activates protein kinase A (PKA) and its downstream targets (p38 mitogen-activated protein kinase [p38 MAPK], activating transcription factor 2 [ATF2], and extracellular signal-regulated kinase 1/2 [ERK1/2]) which subsequently increases the expressions of browning markers (uncoupling protein 1 [UCP1], peroxisome proliferator-activated receptor gamma co-activator 1-alpha [PGC-1α], and PR domain containing 16 [PRDM16]). Histograms show triple independent experiments for immunoblotting analysis and polymerase chain reaction analysis, and are presented as the mean ± standard deviation. Differences between groups were determined using the Statistical Package for the Social Sciences (SPSS, version 17.0; SPSS Inc., Chicago, IL, USA) to perform ANOVA followed by Tukey's post-hoc tests or Student's *t*-test. Statistical significance between control and levodopa treated 3T3-L1 cells is indicated by *$p < 0.05$ or **$p < 0.01$. Three-dimensional structure of L-dopa (B), docked with dopamine receptor 1 (DRD1) (C) to form strong binding complex with 1H bond (D). L-dopa binds with β3-AR (E) to form the docked complex with weak forces (F). ANOVA: analysis of variance.

recognized for their roles in defining the fates of adipocyte precursor cell lineages, as well as on the development and function of brown adipocytes [32]. Overall, our findings indicate that DA and L-Dopa stimulate browning of 3T3-L1 adipocytes by enhancing the expressions of these brown fat-specific marker genes and proteins.

AMPK is known as the master metabolic regulator in all eukaryotes, due to its inherent property to regulate cellular energy homeostasis in a variety of tissues, including the brain. It is also responsible for the rapid regulation of ACC as well as driving lipid mobilization by the direct phosphorylation of HSL and ATGL [33-36]. A previous study demonstrates that AMPK activation mediates DA neuronal atrophy, and its blockage remarkably reduces brain atrophy [37], thereby implying that AMPK activation is widespread in many neurological illnesses, including PD. We postulated

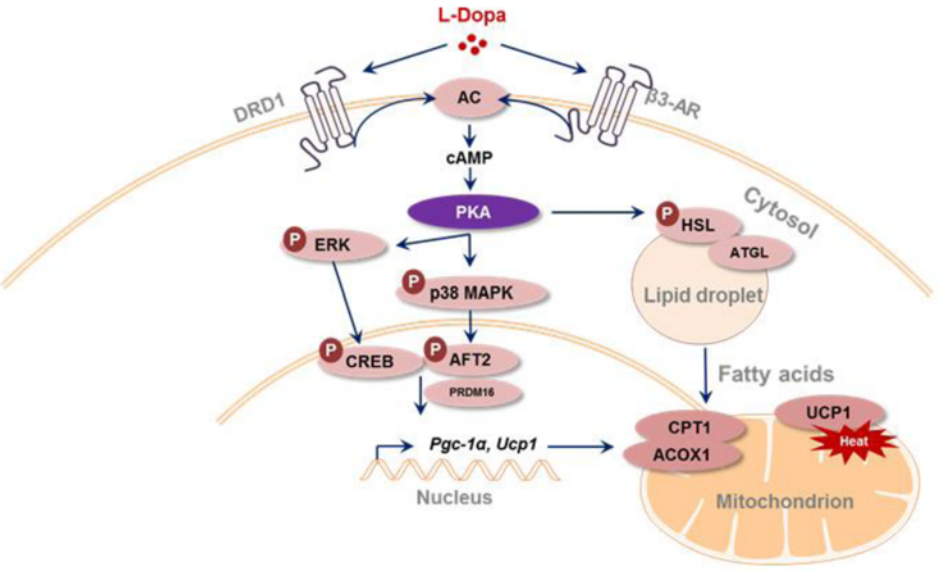

Fig. 8. Suggested molecular mechanism for levodopa (L-Dopa)-induced fat browning in 3T3-L1 white adipocytes. Straight arrows refer to stimulation. DRD1: dopamine receptor D1, β3-AR: beta-3 adrenergic receptor, AC: Adenylyl cyclase, cAMP: Cyclic adenosine monophosphate, PKA: protein kinase A, ERK: extracellular signal-regulated kinase, p38 MAPK: p38 mitogen-activated protein kinase, ATF2: activating transcription factor 2, PRDM16: PR domain containing 16, UCP1: uncoupling protein 1, PGC-1α: peroxisome proliferator-activated receptor gamma co-activator 1-alpha, HSL: hormone-sensitive lipase, ATGL: adipose triglyceride lipase, CPT1: carnitine palmitoyltransferase 1, ACOX1: acyl-coenzyme A oxidase 1.

that L-Dopa could play a role in AMPK upregulation, and consequently reduce the expression of lipogenesis markers (*e.g.*, ACC and FAS) when 3T3-L1 white adipocytes are treated with L-Dopa.

Catecholamines, which include dopaminergic and β-adrenergic neurotransmitters, are known to play an important role in regulating the "fight or flight" response, and have varying effects on thermogenesis. For example, treatment of adipocytes with DA causes an increase in UCP1 levels as well as an induction of lipolysis, thereby elevating FFA levels [29,38]. Our findings are consistent with this report, in which exposure of white adipocytes to L-Dopa induces β-oxidation of fatty acids by upregulating the expressions of vital mitochondrial proteins such as CPT1 and ACOX1. In agreement with our current findings, a previous study reported that postmortem analysis of fatty acid profiles in the brain cortex of PD patients and a non-human primate using gas chromatography revealed that relative concentrations of brain fatty acids are associated with L-Dopa treatment [39]. However, contrary to our findings, another study reported that a combination of L-Dopa and benserazide (carbidopa equivalent) fails to activate lipolysis in adipose tissue [40].

There exists a wealth of information demonstrating that dopaminergic signaling in the hypothalamus plays an important role in the regulation of food intake and energy homeostasis, as DA signaling is a crucial part of the brain reward system and can affect feeding behavior [41,42]. However, little is known about the physiological role in adipocytes [43]. In this study, we determined that L-Dopa activates both DRD1 and β3-AR, thereby consequently increasing the expressions of its downstream molecules such as PKA, p38 MAPK, and ERK, CREB and ATF2 to induce browning in 3T3-L1 white adipocytes.

Studies on the effects of agonism and antagonism of DR have yielded mixed findings related to weight gain, and DRD2 has been more extensively studied than DRD1 [41,44-47]. A recent report demonstrates that DRD1 or DRD2 agonists do not exert lasting and physiologically relevant effects on BAT thermogenesis in mice after peripheral administration, suggesting that both DRD1 and DRD2 in interscapular brown adipose tissue are unlikely to constitute targets for obesity treatment via BAT activation [48].

In the present study, we analyzed the day-dependent expression of DRD1 receptor in 3T3-L1 adipocytes, and found that DRD1 is highly expressed during the differentiation stage. This is in line with the findings of [43] who showed that DRD1 is substantially expressed in differentiated adipocytes. Our mechanistic study showed that L-Dopa treatment simulates browning of 3T3-L1 adipocytes through bi-directional pathways, *viz*., DRD1 and β3-AR, in which PKA/p38 MAPK and ERK were found to be common downstream targets of DRD1 and β3-AR [43,49]. It has also been broadly reported that p38 MAPK potentiates browning and BAT activation through UCP1 transcription

via the activation of CREB, ATF2, and PGC-1α [50]. A recent study supports our findings by demonstrating that CREB is not only downstream of p38 MAPK, but also of ERK [51]. In fact, our group has extensively reported on these downstream signaling pathways for induction of fat browning mediated by numerous compounds, reaffirming that they are likewise downstream targets of β3-AR stimulation [9-11,52,53]. The activation of these downstream targets, in response to stimulation of their respective upstream receptors, confirms the importance of the interaction between dopaminergic and adrenergic systems in the central nervous system (CNS). An earlier study demonstrated the activation of β3-AR in rat adipocytes in response to DA, suggesting that DA activates β3-AR to lower glucose uptake in rat white adipocytes which lack the dopaminergic receptors [54]. Similarly, our present findings indicate that L-Dopa stimulates β3-AR and the relevant downstream targets in 3T3-L1 adipocytes.

Reports on the cross interaction between adrenergic and dopaminergic receptors indicates that these classical neurotransmitters share the same synthesis pathway and are possibly co-localized in some parts of the CNS [55]. Furthermore, these neurotransmitters interact with their specific receptors, and the pharmacology of adrenergic and dopaminergic receptors has been extensively studied [55]. In addition, the exogenous DA activation of β3-AR via an increase of cAMP underscores the interaction between these two receptors [54]. In line with this, our current findings show that L-Dopa stimulates PKA and its downstream targets through the activation of DRD1 and β3-AR, whereas their respective antagonists inhibit these pathways. This suggests that there may be an unexpected interaction between these two receptors, and we are conducting separate research on these receptors.

5. Conclusion

In conclusion, L-Dopa has a remarkable role in browning of 3T3-L1 adipocytes through the activation of both DRD1 and β3-AR, which elucidates the therapeutic potential of L-Dopa in the fight against obesity.

6. Future Perspectives

Our *in vitro* findings may shed light on the action of L-Dopa in the browning of 3T3-L1 adipocytes as part of a clinical approach to prevent obesity. However, further studies need to be conducted to reaffirm the importance of L-Dopa in the mechanisms of obesity prevention and to establish a detailed clinical approach. In addition, it also needs *in vivo* studies using tissue-specific DRD1 knockdown/knockout mice to evaluate the role of L-Dopa on browning of white adipocytes, thereby elucidating the influence of DRD1 on browning mediating pathways in the presence of β3-AR as well as assessment of intracellular ATP levels need to be corroborated.

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Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-021-0361-1) contains supplementary material, which is available to authorized users.

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