

Contents lists available at [ScienceDirect](http://ScienceDirect.com)

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

The neuroprotective role of metformin in advanced glycation end product treated human neural stem cells is AMPK-dependent



Ming-Min Chung^a, Yen-Lin Chen^b, Dee Pei^a, Yi-Chuan Cheng^c, Binggui Sun^d, Christopher J. Nicol^{e,f}, Chia-Hui Yen^g, Han-Min Chen^{h,i}, Yao-Jen Liangⁱ, Ming-Chang Chiang^{i,*}

^a Department of Internal Medicine, Cardinal Tien Hospital, School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan

^b Department of Pathology, Cardinal Tien Hospital, School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan

^c Department of Biochemistry and Molecular Biology, Chang Gung University, Tao Yuan 333, Taiwan

^d Institute of Neuroscience, Zhejiang University School of Medicine, Zhejiang, China

^e Department of Pathology and Molecular Medicine, Cancer Biology and Genetics Division, Cancer Research Institute, Queen's University, Kingston, ON K7L 3N6, Canada

^f Department of Biomedical and Molecular Sciences (Pharmacology and Toxicology), Queen's University, Kingston, ON K7L 3N6, Canada

^g Department of International Business, Ming Chuan University, Taipei 111, Taiwan

^h Graduate Institute of Applied Science and Engineering, Fu Jen Catholic University, New Taipei City 242, Taiwan

ⁱ Department of Life Science, Fu Jen Catholic University, New Taipei City 242, Taiwan

ARTICLE INFO

Article history:

Received 4 September 2014

Received in revised form 5 January 2015

Accepted 8 January 2015

Available online 13 January 2015

Keywords:

AMPK

Metformin

AGEs

hNSCs

ABSTRACT

Diabetic neuronal damage results from hyperglycemia followed by increased formation of advanced glycosylation end products (AGEs), which leads to neurodegeneration, although the molecular mechanisms are still not well understood. Metformin, one of the most widely used anti-diabetic drugs, exerts its effects in part by activation of AMP-activated protein kinase (AMPK). AMPK is a critical evolutionarily conserved enzyme expressed in the liver, skeletal muscle and brain, and promotes cellular energy homeostasis and biogenesis by regulating several metabolic processes. While the mechanisms of AMPK as a metabolic regulator are well established, the neuronal role for AMPK is still unknown. In the present study, human neural stem cells (hNSCs) exposed to AGEs had significantly reduced cell viability, which correlated with decreased AMPK and mitochondria associated gene/protein (PGC1 α , NRF-1 and Tfam) expressions, as well as increased activation of caspase 3 and 9 activities. Metformin prevented AGEs induced cytochrome c release from mitochondria into cytosol in the hNSCs. Co-treatment with metformin significantly abrogated the AGE-mediated effects in hNSCs. Metformin also significantly rescued hNSCs from AGE-mediated mitochondrial deficiency (lower ATP, D-loop level, mitochondrial mass, maximal respiratory function, COX activity, and mitochondrial membrane potential). Furthermore, co-treatment of hNSCs with metformin significantly blocked AGE-mediated reductions in the expression levels of several neuroprotective genes (PPAR γ , Bcl-2 and CREB). These findings extend our understanding of the molecular mechanisms of both AGE-induced neuronal toxicity, and AMPK-dependent neuroprotection by metformin. This study further suggests that AMPK may be a potential therapeutic target for treating diabetic neurodegeneration.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Type 2 diabetes mellitus (DM) is one of the most common metabolic and chronic diseases worldwide, with a prevalence that increases with age and obesity [1]. DM is a disorder that impairs several organ systems, including the brain, and is implicated as a risk factor for Alzheimer's disease (AD) [2–4]. Patients with DM present with cognitive deficits associated with reduced performance on multiple domains of cognitive function [5], as well as structural brain changes [6,7]. Magnetic

Resonance Imaging (MRI) has also demonstrated that subjects with DM have hippocampal and amygdala atrophy relative to control subjects [8]. In addition, population-based studies suggest a link between DM and AD, with the incidence of AD as much as two to five times higher in diabetic patients [5,6]. However, the underlying pathological mechanisms that may associate DM and neurodegeneration are not yet well defined.

Chronic hyperglycemia of the brain may be one of the critical factors involved in neuronal impairment in people with irregular glucose metabolism [9,10]. Several studies show that hyperglycemia leads to slow progressive structural and functional abnormalities in the brain [7,11,12]. Hyperglycemia is accompanied by accelerated formation of AGEs, which accumulate in various tissues during normal aging and at an increased rate in DM patients [13]. A recent study reported that AGEs play a critical role in the pathogenesis of diabetic complications

* Corresponding author at: Department of Life Science, Fu Jen Catholic University, New Taipei City 242, Taiwan. Tel.: +886 2 29052467; fax: +886 2 29052193.

E-mail address: cmcpd@gmail.com (M.-C. Chiang).

and neurodegenerative disorders, including AD [14]. Furthermore, in vitro dose-dependent AGE-mediated suppression of the proliferation and differentiation responses of rat neural stem cells (NSCs) [15], suggests that abnormal blood glucose may be similarly toxic in the brain.

Metformin is a widely prescribed oral anti-hyperglycemic drug for the clinical treatment of DM [16,17]. Metformin exerts its protective anti-diabetic effects in part by activating AMP-activated protein kinase (AMPK) [18–20] and, interestingly, also inhibits glycation reactions [16, 17,21]. AMPK plays a role in regulating glucose and lipid metabolism, senses metabolic stress and integrates diverse physiological signals to restore energy balance [22–24]. AMPK also serves as a regulator of cell survival or death in response to pathological hypoxia, osmotic and oxidative stress [25–28]. Therefore, AMPK plays a key role in intracellular metabolism and is an attractive therapeutic target, especially for energy related diseases [29,30]. AMPK was previously implicated in aging, AD and Parkinson's disease [31–34]. Most importantly, AMPK has a crucial role in hyperglycemia associated with Type 2 DM, neuroprotection, anti-inflammation and alteration of oxidative stress [35,36].

The potential relationship between AGEs and AMPK signaling in diabetic neuropathy has not been studied extensively. In this study, hNSCs treated with AGEs, with or without metformin, were examined for changes in cell death and mitochondrial dysfunction. Our findings provide important new evidence of the mechanisms by which AGEs mediate hNSC degeneration, and metformin confers AMPK-dependent neuroprotection. Together, these data support AMPK as a potential therapeutic drug target to treat neurodegeneration in diabetic patients.

2. Materials and methods

2.1. Cell culture

GIBCO® human neural stem cells (hNSCs) were originally obtained from the National Institutes of Health (NIH) approved H9 (WA09) human embryonic stem cells. Complete StemPro® NSC serum free medium (SFM) was used for optimal growth and expansion of GIBCO® hNSCs, and kept the hNSCs undifferentiated as described previously [37]. Complete StemPro® NSC SFM consists of KnockOut™ D-MEM/F-12 with 2% StemPro® Neural Supplement, 20 ng/ml of EGF, 20 ng/ml of bFGF, and 2 mM of GlutaMAX™-I.

The day before treatment, cells were seeded onto a 35-mm dish at a density of 2×10^5 cells per well. For treatment, cells were grown in wells for 24 h and then exposed to AGEs (0.5 mg/ml) for 24 h in NSC SFM. Next, cells were washed with PBS, followed by one more wash with NSC SFM, and were cultured with the indicated reagents (1 mM metformin or 10 μ M Compound C) for an additional 48 h in NSC SFM. Experimental groups to be conducted are listed below. hNSCs with no treatment were then cultured in NSC SFM for 72 h; hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then cultured in NSC SFM for another 48 h; hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with 1 mM metformin for another 48 h; hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with 1 mM metformin and 10 μ M Compound C for another 48 h.

2.2. Evaluation of cell growth

Cell viability was assayed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, Austin, TX, USA) absorbance and cell count as reported elsewhere [38]. Synchronized hNSCs were treated with AGEs (0.5 mg/ml), AMPK agonist metformin (1 mM) or AMPK pharmacological inhibitor Compound C (10 μ M) for 3 days as indicated. MTT absorbance in solubilized cells was measured at 570 nm using an EZ Read 400 ELISA Reader (Biochrom, Holliston, USA). The cell growth rate was expressed as a percentage of values obtained in vehicle control groups. Metformin and Compound C were purchased from Cayman Chemicals (Ann Arbor, MI, USA). AGE is an abbreviation of Advanced Glycation End product (AGE)-BSA obtained from BioVision (Milpitas,

CA, USA). Aminoguanidine was obtained from Sigma (Saint Louis, MO, USA).

2.3. Caspase activity assay

Caspase activity assay was carried out using caspase-3-like (DEVD-AFC) and caspase-9-like (LEHD-FMK) Fluorometric Protease Assay Kits (Chemicon, Michigan USA) as reported elsewhere [39]. Briefly, cells were homogenized in a lysis buffer for 10 min. The cellular lysate (standardized to protein concentration) was incubated with an equal volume of $2 \times$ reaction buffer (with 0.01 M of dithiothreitol) for an additional 1 h at 37 °C and either caspase-3 (DEVD-AFC) or caspase-9 (LEHD-FMK) substrate at a final concentration of 50 μ M. The fluorescence was measured by a microplate reader with an excitation filter of 390 ± 22 nm and an emission filter of 510 ± 10 nm.

2.4. Western blot assays

Equal amounts of protein were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The resolved proteins were electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) for Western blot analyses as reported elsewhere [40]. Primary antibodies of cytochrome c (1:1000; GeneTex, Inc, Irvine, CA), voltage-dependent anion channel (1:2000; GeneTex), AMPK (1:2000; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (Thr172) AMPK (1:1000; Cell Signaling Technology), PGC1 α (1:2000; GeneTex), phosphorylated PGC1 α (1:1000; GeneTex), NRF-1 (1:2000; GeneTex), Tfam (1:2000; GeneTex), and actin (1:3000; GeneTex) were utilized according to manufacturer's instructions.

2.5. Measurements of cytochrome c release

For measurement of cytochrome c release, mitochondria and cytosolic fractions were prepared from hNSCs. Briefly, cells were washed with cold PBS and resuspended in ice-cold buffer (10 mM Hepes, 1 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 1 mM Na₃VO₄, 10 mM NaF, 1 μ M okadaic acid, 0.5% Nonidet P-40 and protease inhibitor-complete cocktail). After 30 min of incubation on ice, cells were homogenized (Dounce, 20 strokes) in buffer. Homogenates were then centrifuged at 650 g for 5 min at 4 °C, and the supernatants were re-centrifuged at 9000 g for 30 min at 4 °C to collect the mitochondrial fraction. The supernatants were again centrifuged at 95,000 g for 1 h at 4 °C, and the final supernatant was used as a cytosolic fraction. Cytochrome c levels in the mitochondrial and cytosolic fraction were determined by Western blot analysis.

2.6. RNA isolation and quantitative real-time polymerase chain reaction (Q-PCR)

Total RNA was isolated, and reverse-transcribed into cDNA as described previously [41]. A real-time quantitative PCR was performed using a TaqMan kit (PE Applied Biosystems, Foster City, CA, USA) on a StepOne quantitative PCR machine (PE Applied Biosystems) using heat-activated TaqDNA polymerase (Amplitaq Gold; PE Applied Biosystems). The sequences of primers were as follows: AMPK (5'-GGGTGAAGA-TCGGACACTACGT-3' and 5'-TTGATGTTCAATCTCACTTG-3'), PGC1 α (5'-TGAGAGGGCCAAGCAAAG-3' and 5'-ATAAATCACA CGGCGCTCTT-3'), NRF1 (5'-CCATCTGGTGGCTGAAG-3' and 5'-GTGC CTGGTCCATGAAA-3'), Tfam (5'-GAACAAC TACCCATATTTAAAGCTCA-3' and 5'-GAATCAGGAAGT-TCCTCCA-3'), D-loop (5'-GGTCTTACTTC AGGGCCATCA-3' and 5'-GATT-AGACCCGTTACCATCGAGAT-3'), PPAR γ (5'-AAAGAAGCCGACACTAAACC-3' and 5'-CTTCCATTACGGAGAGATCC-3'), Bcl-2 (5'-ACTTTGCAGAGATGTCC-AGT-3' and 5'-CGGTTACAGGTAC TCAGTCAT-3'), CREB (5'-CCAAGCTTATGG-ATCCTCTGGAGAGAAGA TGG-3' and 5'-GCCTCGAGAAGCACATTGACGCT-CCTGAC-3'), RAGE (5'-

CACACTGCAGTCGGAGCTAA-3' and 5'-GCTACTGCT-CCACCTTCTGG-3'), and GAPDH (5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3'). Independent reverse-transcription PCRs were

performed as described previously [41]. Relative target gene transcript levels were calculated using standard curves of serial RNA dilutions, and normalized to GAPDH levels of the same RNA sample.

2.7. Measurement of intracellular ATP concentration

To determine ATP levels, hNSCs were collected in a lysis buffer (0.1 M Tris, 0.04 M EDTA, pH 7.2), and boiled for 3 min. Samples were then centrifuged (112 g for 5 min) and supernatant protein concentrations were determined using a Bradford assay (Promega, Madison, WI, USA). The supernatants were assessed using a luciferin/luciferase assay as described previously [38]. Sample ATP levels were normalized to protein content. The reaction buffer for this assay contained 60 μ M of luciferin, 0.06 μ g/ml of luciferase, 0.01 M of magnesium acetate, 0.05% of bovine serum albumin, and 0.2 M of Tris (pH 7.5).

2.8. Mitochondrial mass

The fluorescent probe Mitotracker Green™ dye (MitoGreen, Invitrogen, Carlsbad, CA, USA) which binds mitochondrial membrane lipids regardless of mitochondrial membrane potential or oxidant status, was used to determine the mass of mitochondria as previously described [39]. Briefly, cells were loaded with 0.2 μ M/ml of Mitotracker Green™ dye in the medium for 30 min at 37 °C. Fluorescence measurements were made with excitation at 490 nm and emission at 516 nm using fluorescence microscopy (OPTIMA G-303), and reported as the mean of fluorescent signals.

2.9. Mitochondrial functional parameters

For mitochondrial respiratory studies cells were grown on a 10 cm plate, treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h, then trypsinized, and suspended in 0.5 ml of mitochondrial isolated buffer. Respiratory measurements of mitochondria were isolated using the mitochondria isolation kit utilized according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). 50 μ g of mitochondria were suspended in a sealed and continually stirred at 37 °C containing 0.3 ml of respiration buffer (100 mM KCl, 2 mM MgCl₂, 4 mM KH₂PO₄, 10 mM pyruvate, 5 mM malate, 250 μ M EGTA, 10 mM HEPES). The maximal respiratory rate was gained following the addition of 10 μ M FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone). The respiratory function of isolated mitochondria (0.2 mg/ml final concentration) was measured using a miniature Clark-type oxygen electrode (MT200 Mitocell Miniature Respirometer, Hamden, CT, USA).

The cytochrome oxidase (COX) activity was measured using the assay kit from Sigma (St. Louis, USA) using isolated mitochondrial fractions from the isolation kit (Thermo Fisher Scientific) for hNSCs. The mitochondrial fraction (2 μ g) was added to 1 ml of the reaction solution, then assayed with the COX activity assay; reactions were set up following the procedures provided by the manufacturer. The absorbance

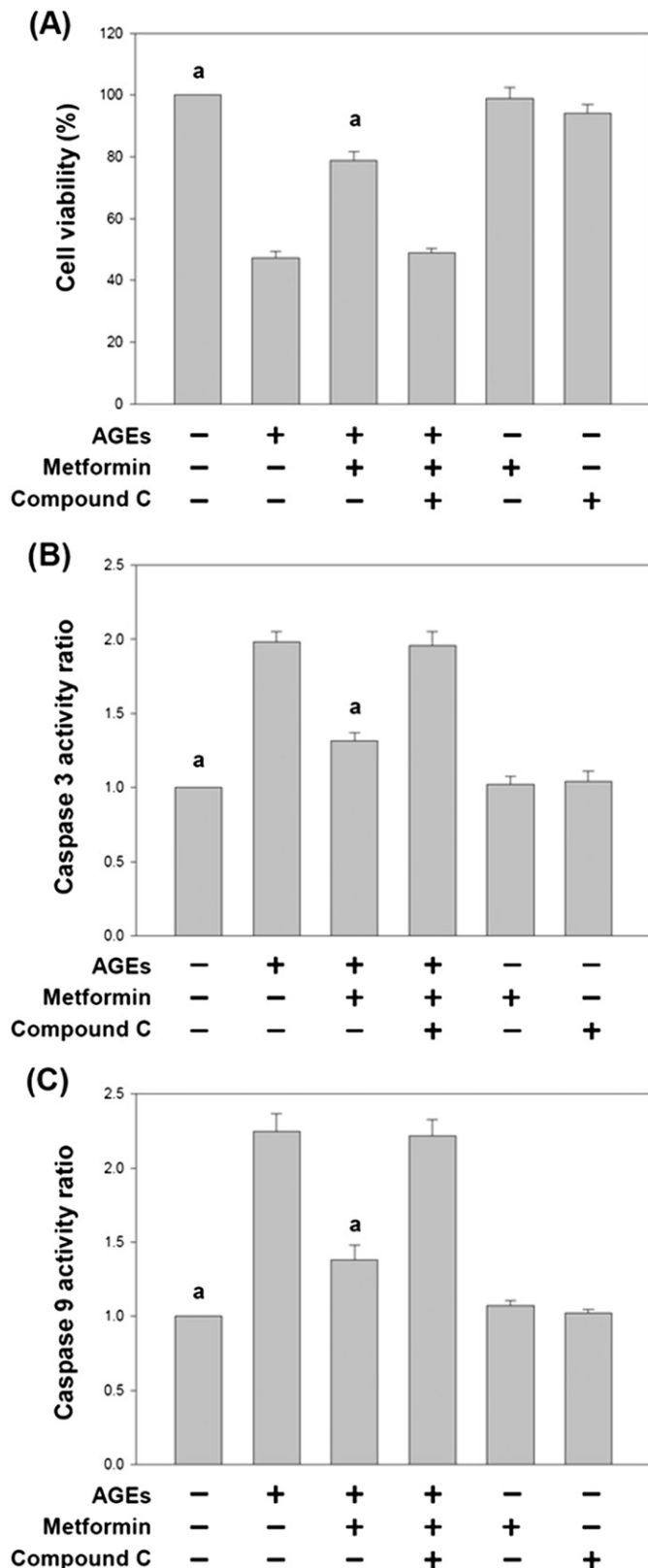


Fig. 1. Metformin rescues the AGE-mediated decrease in hNSC viability via the AMPK pathway. (A) hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h, and cell viability was detected by MTT assay. All reactions were run in triplicate from each independent experiment. Value A is expressed as a percentage of the indicated transcript in CON and is presented as the mean \pm SEM values from three independent experiments. (B, C) Caspase activities were detected by a fluorometric protease assay using substrates for caspase-3-like (DEVD-AFC) and caspase-9-like (LEHD-FMK). All reactions were run in triplicate from each independent experiment. Values B and C are expressed as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

readings were detected by a spectrophotometer at 550 nm and COX activity was measured as unit/mg of mitochondrial protein.

The measurement of mitochondrial membrane potential was detected using the JC-1 dye from Life Technologies (Waltham, USA) for hNSCs. Briefly, cells were treated with 1 μ M JC-1 for 30 min in Earle's balanced salt solution (EBSS) at 37 °C. The cells were then washed three times in EBSS before fluorescence values were detected. The fluorescence was calculated by a microplate reader with an excitation filter of 530 ± 25 nm and an emission filter of 590 ± 30 nm.

2.10. Cell transfection

One day before transfection, hNSCs were seeded onto a 35-mm dish at a density of 2×10^5 cells per well. Cells were transfected with 1 mg of PGC1 α siRNA (Human sc-38884, Santa Cruz Biotechnology) using Lipofectamine RNAiMAX (Invitrogen) in medium for 24 h, and then treated with the desired reagent for another 48 h as reported elsewhere [38]. Experimental groups to be conducted are listed below. hNSCs with no treatment were then cultured in NSC SFM for 72 h; hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then cultured in NSC SFM for another 48 h; hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with 1 mM metformin for another 48 h; hNSCs were transfected with PGC1 α siRNA and treated with AGEs (0.5 mg/ml) for 24 h, then treated with 1 mM metformin for another 48 h.

2.11. Statistical analysis

All reactions were run in triplicate from each independent experiment. All data were expressed as means \pm SEM from three independent experiments. To establish significance, data were subjected to unpaired one-way ANOVA using the Sigma Stat 3.5 software statistical package (Systat SigmaStat V3.5.0.54 Software; San Jose, California, USA). The criterion for significance was set at $p < 0.001$. Differences between groups were assessed with Student's *t* tests or one-way analysis of variance (one-way ANOVA) as indicated.

3. Results

3.1. Metformin rescued cell viability in hNSCs treated with AGEs via the AMPK pathway

The effects of AGEs on cell viability and caspase 3/9 (a marker of caspase cascade activation) activity in hNSCs were initially assessed. Compared to vehicle controls, hNSCs treated with AGEs (0.5 mg/ml) for 72 h had significantly reduced cell viability ($p < 0.001$) (Fig. 1A). In addition, hNSC caspase 3 and 9 activities, detected after AGE (0.5 mg/ml) treatment for 72 h, were significantly increased 2-fold compared to their respective controls ($p < 0.001$) (Fig. 1B, C). Furthermore, treatment with an AMPK agonist (metformin) significantly ($p < 0.001$) normalized both cell viability (Fig. 1A) and caspase 3/9 activities (Fig. 1B, C), although this protective effect was blocked by co-treatment with an antagonist of AMPK (Compound C). To test the toxic effects of internal controls (metformin and Compound C, alone), we respectively treated

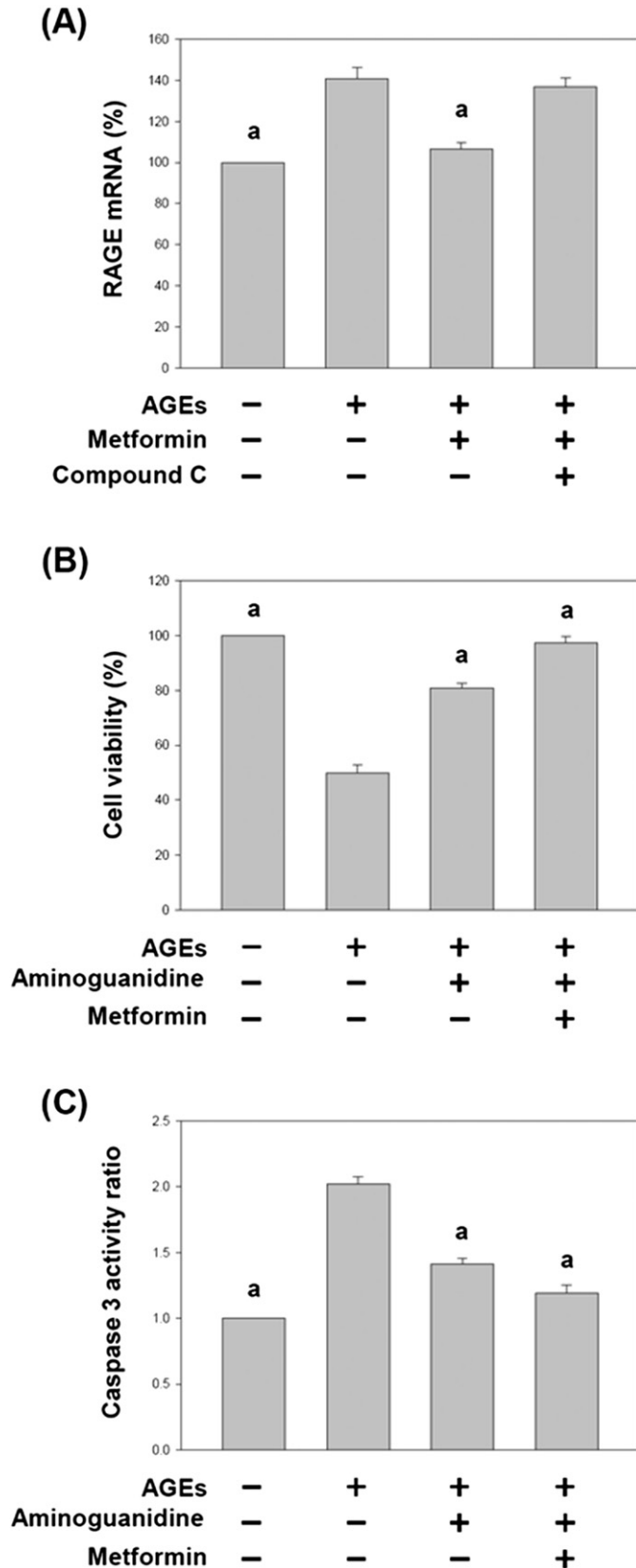


Fig. 2. Effects of RAGE and aminoguanidine on hNSCs. (A) hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h. RAGE transcript in the indicated hNSCs was analyzed using the Q-PCR technique. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR technique of the indicated gene was performed and normalized to that of GAPDH. (B, C) hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (20 μ M aminoguanidine or 1 mM metformin) for another 48 h. (B) Cell viability was detected by MTT assay. (C) Caspase activities were detected by a fluorometric protease assay using substrates for caspase-3-like (DEVD-AFC). All reactions were run in triplicate from each independent experiment. Values A, B and C are expressed as a percentage of the indicated transcript in CON and are presented as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

them with metformin or Compound C in hNSCs, and then measured the level of cell viability, caspase 3 and 9. We found that hNSCs treated with metformin or Compound C for 72 h showed no effect on the level of cell viability, caspase 3 and caspase 9 (Fig. 1).

Several results of studies suggest that AGE receptors (RAGE) play important roles in the pathogenesis of diabetic neurodegeneration [42–44]. To verify whether AGEs induce the neurotoxicity through RAGE, we first measured the level of RAGE after AGE treatment. Consistent with the effect of AGEs on the level of RAGE [45], our results suggest that treatment by metformin suppressed the AGE-induced upregulation of RAGE mRNA levels in hNSCs, which were blocked by Compound C (Fig. 2A). To explore the roles of RAGE, we treated with aminoguanidine, an inhibitor of AGE formation, and that blocked the specific RAGE [46–48]. We found that aminoguanidine treatment improved the level of cell viability (Fig. 2B) and decreased caspase 3 activity (Fig. 2C) in hNSCs with AGEs. Interestingly, we also display that combined treatment (aminoguanidine and metformin) significantly normalized both cell viability and caspase 3 in the hNSCs with AGEs. These data suggest that aminoguanidine and metformin may mediate the AGE–RAGE axis induced toxic effects of neural impairments.

Release of cytochrome c from mitochondria is one of the critical initial steps in the causation of the apoptosis including activation of caspase 9 [49]. To test whether the effect of caspase in AGE induced cell death needs cytochrome c, hNSCs were treated with AGEs. The results exhibited in Fig. 3 distinctly demonstrate that previous to AGE treatment, the mass of cytochrome c is localized in the mitochondria and scarcely discoverable levels were detected in the cytosol. In contrast, incubation of cells to AGEs affected release of cytochrome c from the mitochondria into the cytosol (Fig. 3). Importantly, treatment with metformin significantly prevented cytochrome c from the mitochondria into the cytosol which was blocked by Compound C.

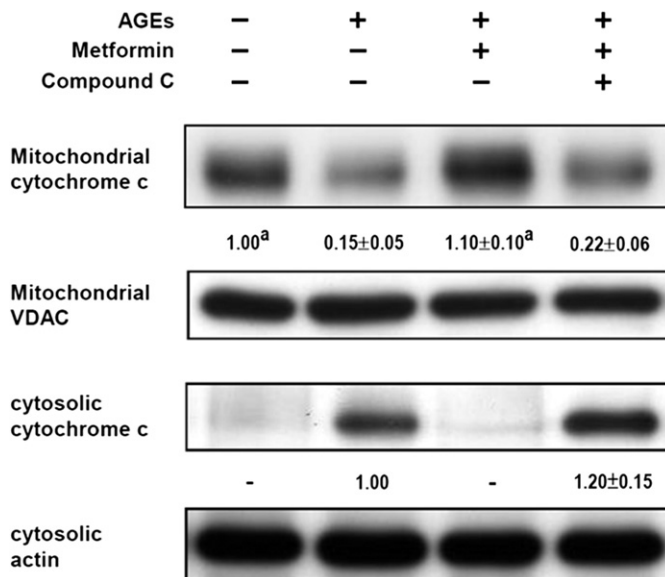


Fig. 3. Metformin prevented AGE induced cytochrome c release from mitochondria into cytosol in the hNSCs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μM Compound C) for another 48 h. Lysates (20 μg) were collected from the indicated treatment group, and subjected to a Western blot analysis. Levels of mitochondrial cytochrome c protein were normalized with the corresponding internal control (voltage-dependent anion channel; VDAC), compared with those in CON, shown at the bottom of the corresponding column. Levels of cytosolic cytochrome c protein were normalized with the corresponding internal control (actin), compared with those in CON, shown at the bottom of the corresponding column. All reactions were run in triplicate from each independent experiment. Values are expressed as a percentage of the indicated transcript in CON and are presented as the mean ± SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

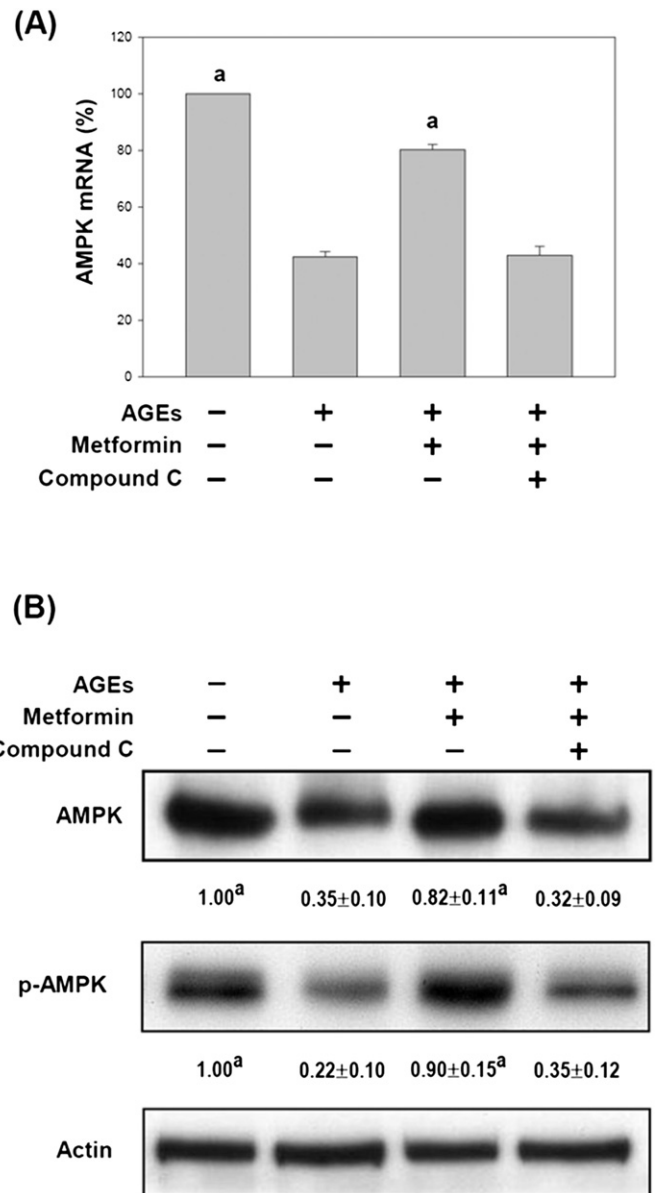


Fig. 4. Co-treatment with metformin restores AMPK expression in the AGE-treated hNSCs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μM Compound C) for another 48 h. (A) The AMPK transcripts in the indicated hNSCs were analyzed using the Q-PCR technique. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR analysis of the indicated genes was performed and normalized to that of GAPDH. (B) Lysates (20 μg) were collected from the indicated treatment group, and subjected to a Western blot analysis. Levels of AMPK and p-AMPK protein were normalized with the corresponding internal control (actin), compared with those in CON, shown at the bottom of the corresponding column. All reactions were run in triplicate from each independent experiment. Values A and B are expressed as a percentage of the indicated transcript in CON and are presented as the mean ± SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

3.2. Metformin rescued AMPK, PGC1α and downstream expression levels suppressed in hNSCs treated with AGEs

Both the transcript and protein levels of AMPK were significantly reduced in hNSCs treated with AGEs compared to vehicle controls ($p < 0.001$) (Fig. 4). In contrast, the addition of metformin significantly enhanced AMPK expression, but this rescue was blocked in the presence of Compound C (Fig. 4). It has been demonstrated that AMPK activation, through the phosphorylation at Thr-172,

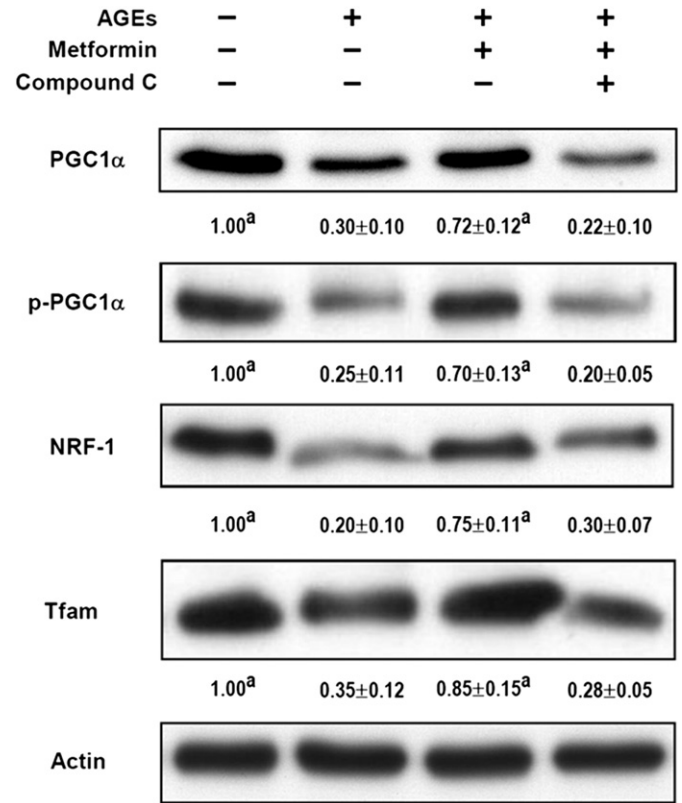
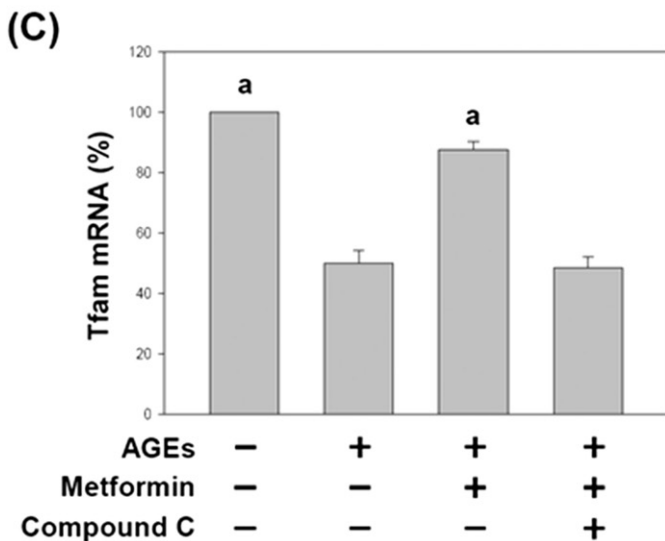
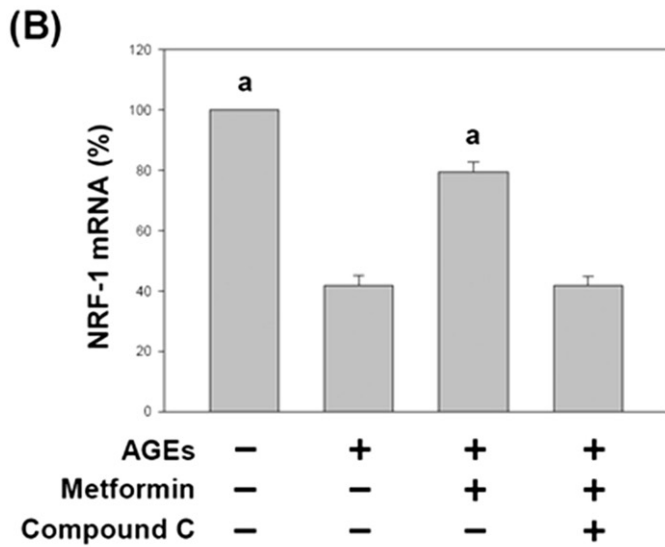
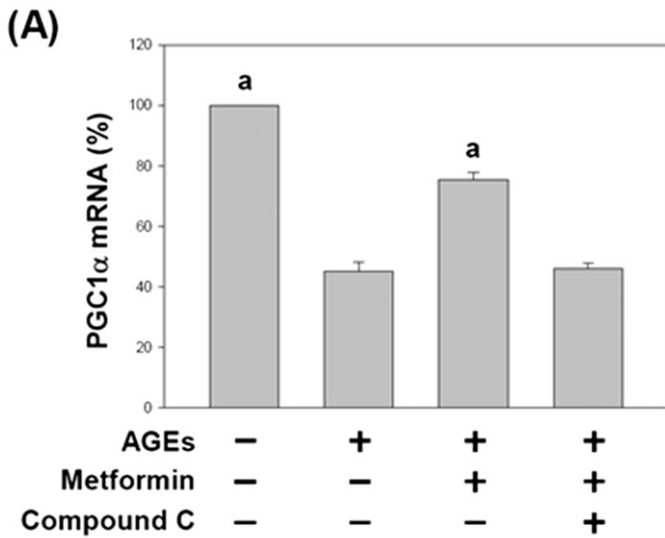


Fig. 6. Metformin enhanced the levels of mitochondrial proteins in the hNSCs treated with AGEs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μM Compound C) for another 48 h. Lysates (20 μg) were collected from the indicated treatment group, and subjected to a western blot analysis. Levels of PGC1α, p-PGC1α, NRF1, and Tfam proteins were normalized with the corresponding internal control (actin), compared with those in CON, shown at the bottom of the corresponding column. All reactions were run in triplicate from each independent experiment. Values are expressed as percentage of the indicated transcript in CON and are presented as the mean ± SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

promotes diverse physiological signals which have been shown to be involved in protective actions [50–52]. In this study we tested whether the AMPK phosphorylation at Thr-172 down-regulation occurring in hNSCs could be in part responsible for the effects of AGEs. Fig. 4B exhibited that treatment with metformin significantly enhanced the protein phosphorylation levels of AMPK at Thr-172, which were blocked by Compound C.

Interestingly, the mRNA transcripts of PPAR coactivator-1α (PGC1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (Tfam) were significantly decreased by more than 50% in AGE-treated hNSCs compared to respective controls ($p < 0.001$) (Fig. 5). Co-treatment with metformin significantly enhanced PGC1α, NRF-1, and Tfam mRNA levels, which were blocked by the presence of Compound C (Fig. 5). To further evaluate

Fig. 5. Metformin improved the expression of mitochondrial genes in the hNSCs treated with AGEs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μM Compound C) for another 48 h. The PGC1α (A), NRF1 (B) and Tfam (C) transcripts in the indicated hNSCs were analyzed using the Q-PCR technique. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR technique of the indicated gene was performed and normalized to that of GAPDH. All reactions were run in triplicate from each independent experiment. Values A, B and C are expressed as percentages of the indicated transcript in CON and are presented as the mean ± SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

the protein levels of PGC1 α , p-PGC1 α , NRF-1, and Tfam in hNSCs, we analyzed the expression by Western blot assay. We found that metformin increased PGC1 α , p-PGC1 α , NRF-1, and Tfam expression in the hNSCs with AGEs, which was blocked by Compound C (Fig. 6).

Indeed, stimulation of AMPK directly phosphorylates the PGC1 α protein, which in turn induces genes associated to mitochondrial function and biogenesis [53,54]. Fig. 6 exhibited that treatment with metformin significantly enhanced the protein phosphorylation levels of PGC1 α , which were blocked by Compound C. In addition, PGC1 α has also been demonstrated in mitochondrial function through its capacity to control a number of genes such as NRF1 and Tfam [38,55]. Fig. 7 illustrated that treatment with metformin significantly enhanced

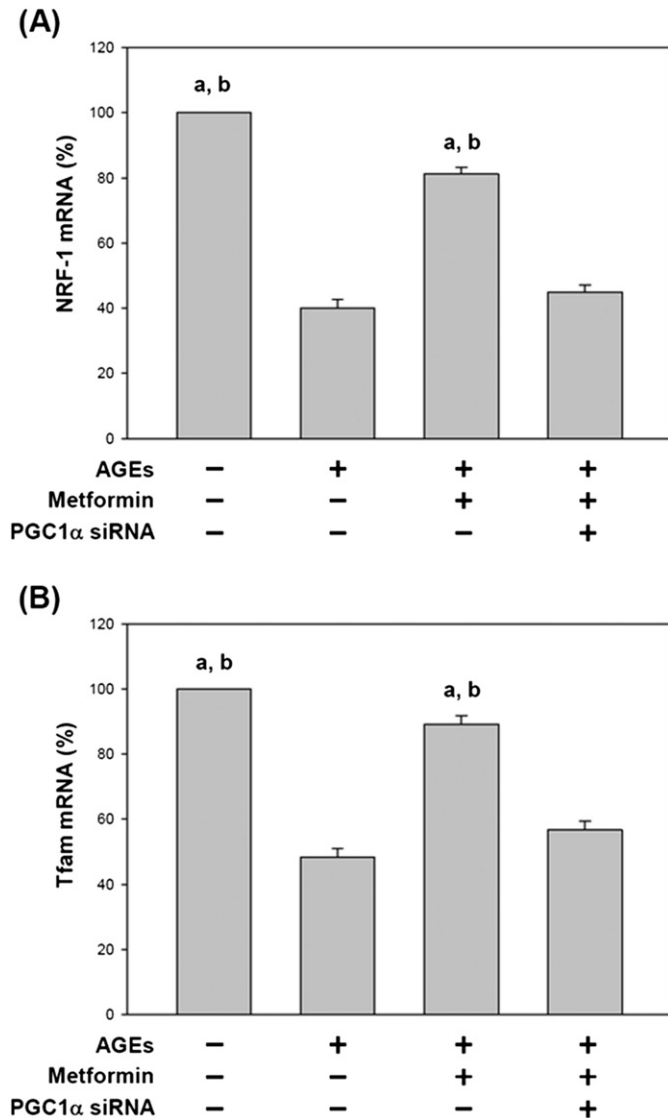


Fig. 7. Effects of metformin on the hNSCs were blocked by PGC1 α siRNA. hNSCs were transfected with 1 mg of PGC1 α siRNA using Lipofectamine RNAiMAX for 24 h, and then treated with the indicated reagent (1 mM metformin) for another 48 h. The NRF1 (A) and Tfam (B) transcripts in the indicated hNSCs were analyzed using the Q-PCR technique. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR technique of the indicated gene was performed and normalized to that of GAPDH. All reactions were run in triplicate from each independent experiment. Values A and B are expressed as percentages of the indicated transcript in CON and are presented as the mean \pm SEM values from three independent experiments. Specific comparison to the indicated hNSCs with AGEs is denoted as 'a' ($p < 0.001$; one-way ANOVA). Specific comparison to the indicated hNSCs with AGEs, metformin and PGC1 α siRNA is denoted as 'b' ($p < 0.001$; one-way ANOVA).

the transcript levels of NRF1 and Tfam, which were blocked by PGC1 α siRNA transfection.

3.3. Metformin enhanced ATP levels, D-loop, mitochondrial mass and mitochondrial functional parameters in AGE-treated hNSCs

To analyze the consequence of potential mitochondrial biogenesis, hNSCs were evaluated using an ATP assay. ATP levels in AGE-treated hNSCs were significantly decreased compared to vehicle controls ($p < 0.001$). However, co-treatment with metformin significantly restored ATP levels to almost normal levels, but only in the absence of Compound C (Fig. 8A). To further evaluate mitochondrial DNA

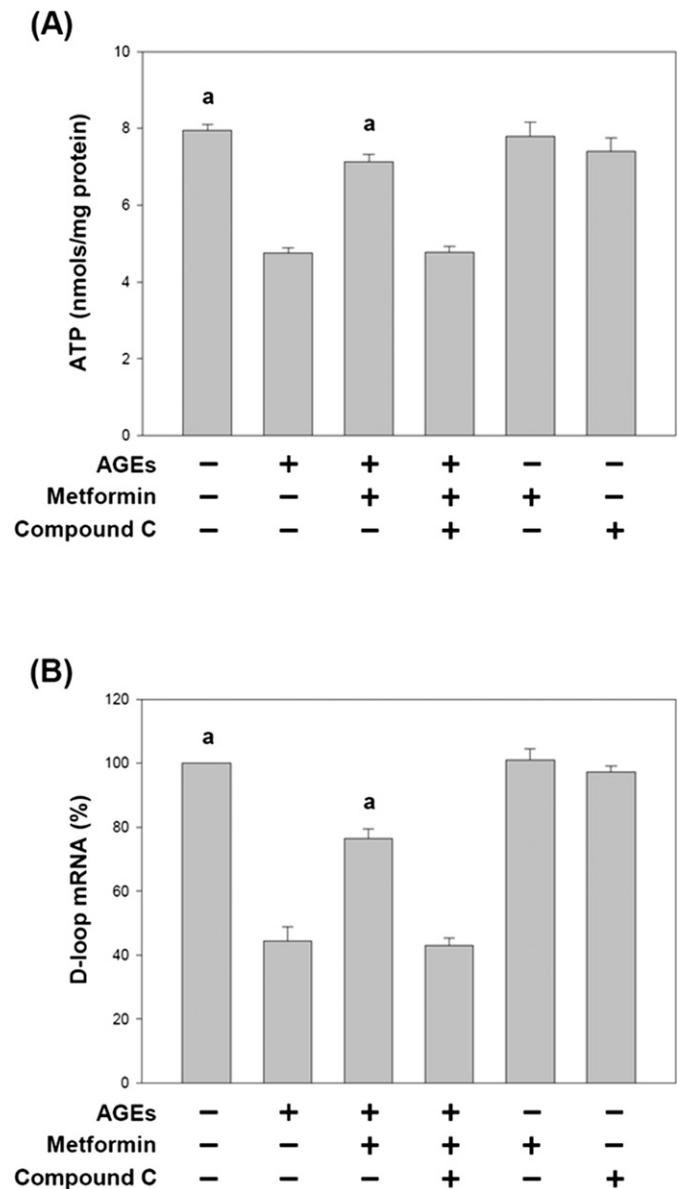


Fig. 8. Metformin enhanced ATP and D-loop levels in AGE treated hNSCs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h. (A) Lysates harvested from the indicated treatment were evaluated by the ATP assay. Data are expressed as the mean \pm SEM values from three independent experiments. (B) The D-loop mRNA levels in the indicated hNSCs were analyzed using Q-PCR. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR technique of the indicated gene was performed and normalized to that of GAPDH. All reactions were run in triplicate from each independent experiment. Values are expressed as percentages of the indicated transcript in CON and are presented as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

(mtDNA) copy number in hNSCs, D-loop mRNA expression [38] was examined by QPCR. Similar to the findings for ATP, D-loop expression levels were significantly decreased in AGE-treated hNSCs ($p < 0.001$) compared to vehicle controls; however, metformin co-treatment restored levels in hNSCs to almost normal, except when in the presence of the AMPK antagonist Compound C (Fig. 8B). To test the effects of internal controls (metformin and Compound C, alone), we respectively treated them with metformin or Compound C in hNSCs, and then measured the level of ATP and D-loop expression. We found that hNSCs treated with metformin or Compound C for 72 h showed no effect on the level of ATP and D-loop expression (Fig. 8).

Stimulation of AMPK promotes mitochondrial biogenesis and remodeling via the induction of PGC1 α and its downstream target genes [55–57]. Therefore, we evaluated whether metformin regulated mitochondrial capacity via an AMPK-dependent pathway in AGE-treated hNSCs. The mitochondrial biogenesis assay using MitoGreen [38,39], and reflective of mitochondrial mass, was used to assess the effects of AGE treatment on hNSCs. As shown in representative images (Fig. 9A), and when quantitated by mean fluorescence (Fig. 9B), these data show that treatment with AGEs significantly decreases hNSC mitochondrial mass by almost 60% compared to vehicle controls ($p < 0.001$), and that co-treatment with metformin significantly abrogated this effect in the absence of Compound C.

To further verify the role of AGEs in mitochondrial functional parameters, we used assays of maximal respiratory function, COX activity, and mitochondrial membrane potential. We found that mitochondrial ability in hNSCs with metformin attenuated the AGE-induced reduction of maximal respiratory function (Fig. 10A), COX activity (Fig. 10B), and mitochondrial membrane potential (Fig. 10C), which were blocked by Compound C. These mitochondrial functional parameters confirm that metformin elevated mitochondrial function in the hNSCs with AGEs via AMPK.

3.4. Metformin increases neuroprotective gene expression levels in AGE-treated hNSCs

To evaluate whether activation of AMPK in hNSCs treated with AGEs exerts a neuroprotective effect, the expression levels of several genes (PPAR γ , Bcl-2 and CREB) that are implicated as important in hNSCs survival were assessed. This is of particular interest because these genes (PPAR γ , Bcl-2 and CREB) are downstream targets of AMPK [58–62]. The mRNA transcript levels of PPAR γ , Bcl-2 and CREB were significantly lower in AGE-treated hNSCs compared to respective vehicle controls ($p < 0.001$) (Fig. 11). Co-treatment with metformin significantly increased transcript levels in hNSCs compared to AGE-treatment alone,

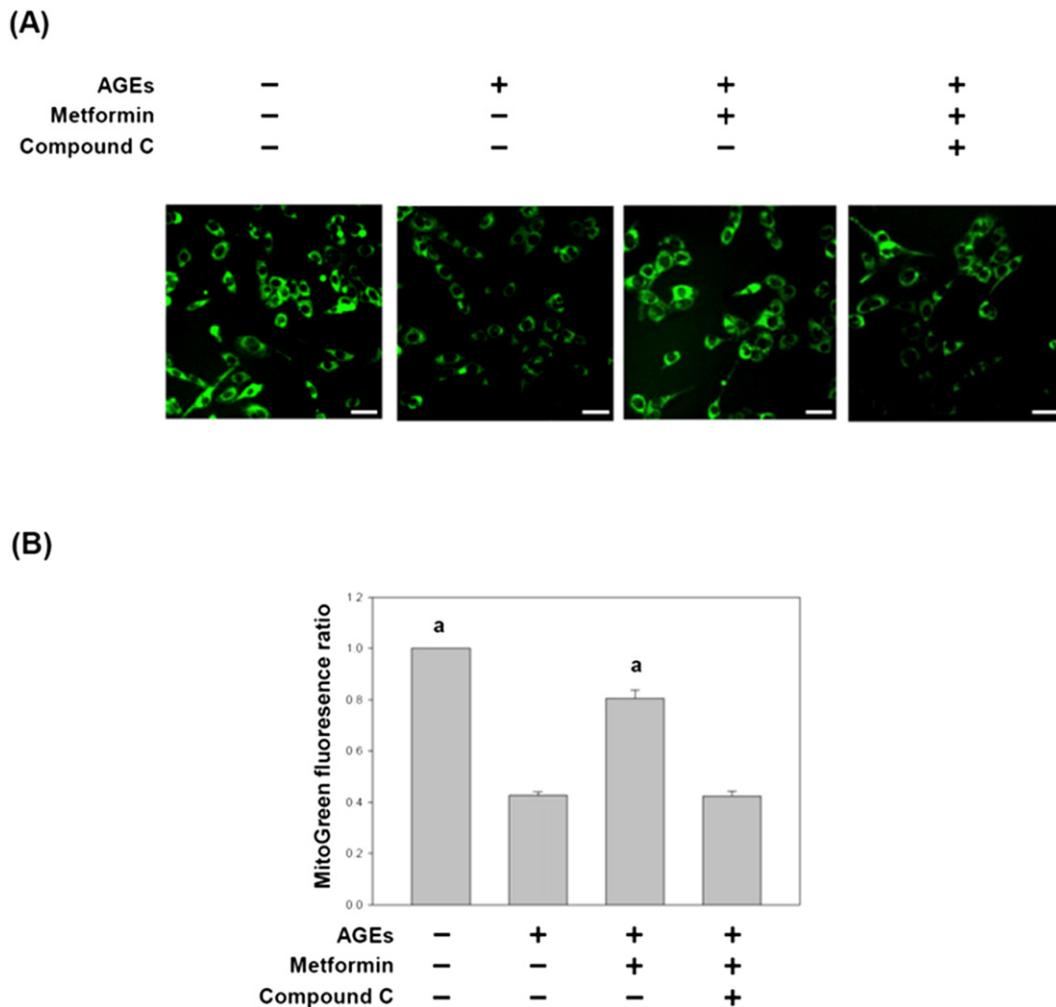
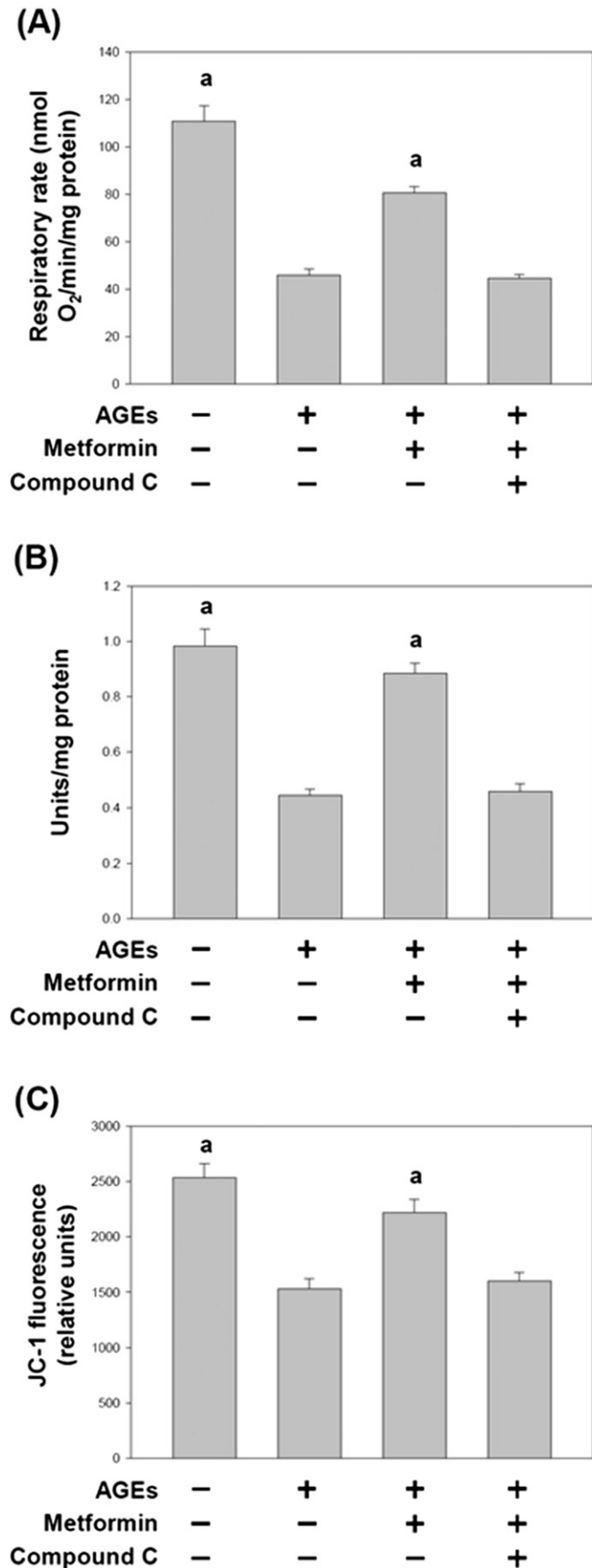


Fig. 9. Metformin increased mitochondrial mass in AGE-treated hNSCs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h. (A) hNSCs were collected to determine the level of mitochondrial mass using Mitotracker Green™ dye (green). Scale bar: 100 μ m. (B) The expression levels of mitochondrial mass were normalized to those of cell numbers. All reactions were run in triplicate from each independent experiment. Values are expressed as a percentage of the indicated transcript in CON and are presented as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).



but this protective effect was absent in the presence of Compound C (Fig. 11).

4. Discussion

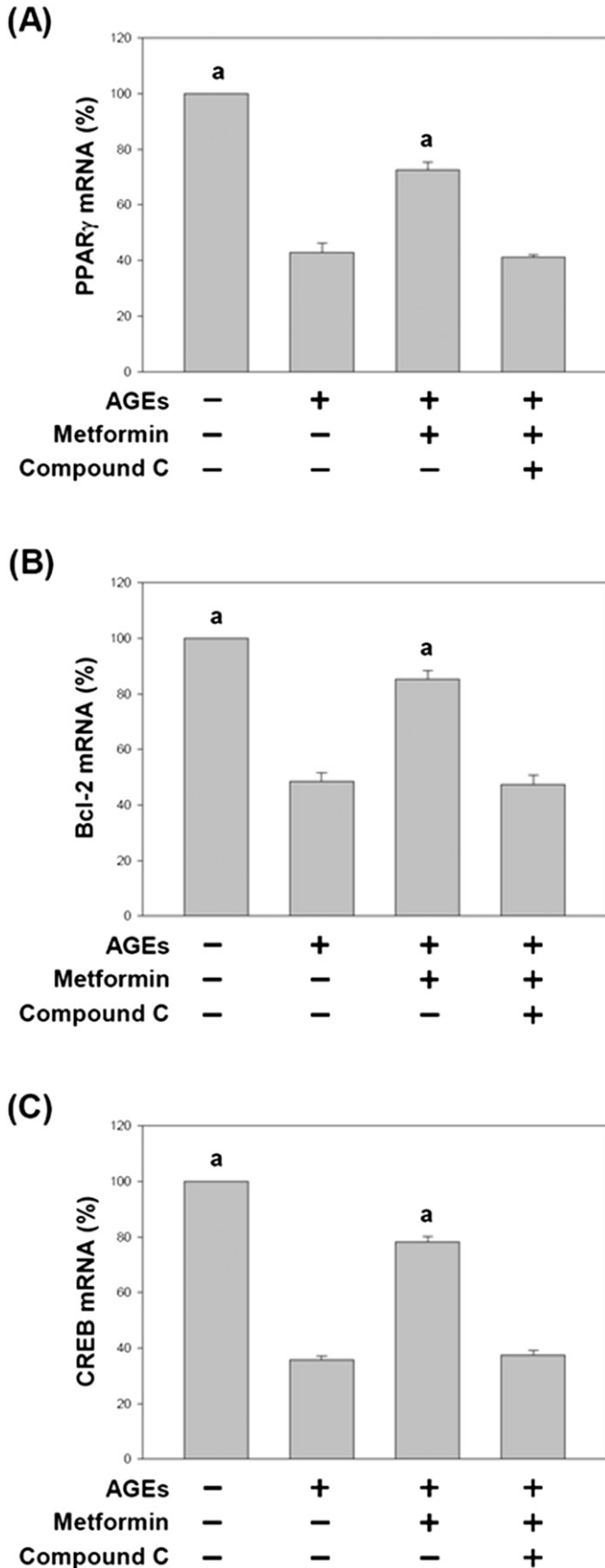
The neuroprotective effects of AMPK activation following AGE-treatment of hNSCs have not been explored previously. In the present study, it is clearly shown that AGEs enhance hNSC cell death and mitochondrial dysfunction via downregulation of AMPK and its downstream signaling pathways. More interestingly, the gold standard AMPK activator metformin significantly protected hNSCs from the deleterious effects induced by AGE exposure in an AMPK-dependent fashion. Because several reports indicated that AGEs decrease mitochondrial capacity [63, 64], and Wareski and colleagues (2009) showed that AMPK stimulation, through the induction of the PGC1 α , promotes mitochondrial function [65], a selected group of gene signaling pathways that may be relevant to these effects was also evaluated. Metformin also enhanced AMPK, PGC1 α , NRF-1 and Tfam expressions in AGE-treated hNSCs, which may contribute to the observed elevation in mitochondrial functions. Moreover, metformin enhanced expression of neuroprotective genes may assist in protecting hNSCs against AGE-induced toxicity. Collectively, these findings strongly implicate AMPK targeting by AGEs in the pathogenesis of neurodegenerative diseases in DM patients, and unveil the first evidence of AMPK expression and signaling as critical to regulate mitochondrial defense systems in hNSCs. These data also raise the possibility that metformin may be a beneficial therapeutic intervention to override AGE-mediated neurodegeneration in diabetic patients.

Humans with poorly managed DM show hyperglycemia, followed by an accelerated rate of AGE formation and accumulation. AGEs in several tissues are known to increase during aging and DM [13]. One study has also demonstrated that AGEs play major roles in the pathogenesis of diabetic neuropathy and neurodegenerative disorders [14]. Importantly, further study may be needed in researching the effects of AGEs on neurodegeneration, and the detailed mechanisms contributing to their pathogenesis. Neuronal apoptosis is observed in the hippocampus of diabetic experimental models in rats [66]. Hence, the studies here further extend our understanding of the chief role of AGE formation and its accumulation in the impairment of cell viability, and support increased AGE levels as risk factors of neuronal impairment in diabetic patients. This study provided direct evidence for the protection of the role of AMPK in the hNSCs with AGEs. Our findings are consistent with other studies showing that metformin treatment, in an AMPK-dependent manner, similarly protected cortical neurons from ethanol-induced apoptotic neurodegeneration [67], and etoposide-induced cell death [68].

Stimulation of the AMPK pathway promoted hNSC proliferation, whereas inhibition of AMPK was correlated with inhibition of hNSC proliferation followed by cellular apoptosis via the caspase cascade. Here, AGE treatment was shown to directly impact on the expression of activated caspases 3 and 9 in hNSCs.

These are the first such studies to unveil the AMPK-dependent neuroprotective effects of co-treatment of metformin in hNSCs exposed to AGEs, and the underlying mechanisms. Kuhla and colleagues previously

Fig. 10. Metformin improved the performance of mitochondrial functions in the hNSCs treated with AGEs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h. (A) Maximal respiratory rate obtained from the indicated treatment were evaluated by the FCCP assay. (B) COX activity was assayed with the cytochrome c Oxidase Assay Kit. (C) hNSCs were collected to determine the level of mitochondrial membrane potential using JC-1 dye. All reactions were run in triplicate from each independent experiment. Values are expressed as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).



reported that AGEs decrease mitochondrial activity and lead to energy depletion [63]. Since AMPK is critical to mitochondrial stabilization [69,70], a decrease in the available AMPK in the hNSCs with AGEs likely contributes to further damage of these mitochondrial functions. Aberrant mitochondrial bioenergetics in DM suggest that abnormal AMPK/PGC1 α signaling in neuronal mitochondrial function may lead to diabetic neuropathy [71]. Moreover, Choi and colleagues (2014) showed that PGC1 α regulated the mitochondrial degeneration in peripheral neurons in diabetic mice [72]. The possible connection between AMPK, PGC1 α and mitochondrial signaling in the hNSCs with AGEs has not been studied extensively. In the present study, activation of AMPK, and subsequently mitochondrial function, was correlated with stimulation of the PGC1 α pathway and up-regulation of mitochondrial genes.

Several papers have suggested that alterations in the function of the mitochondria and neuroprotective effects of AMPK are involved in both neurodegenerative and metabolic diseases [30,73]. In the present study, there is clear evidence that profound mitochondrial impairment is an important event in AGE-mediated toxicity in hNSCs. This is also consistent with another report on the beneficial effects of metformin on mitochondrial biogenesis [74]. It has been explained that AMPK stimulation by metformin, through PGC1 α , stimulates mitochondrial biogenesis. Moreover, activation of AMPK was strongly correlated with upregulation of survival gene (PPAR γ , Bcl-2 and CREB) mRNA expression [58–62]. The neuroprotective effects of metformin were effectively blocked by Compound C, demonstrating that the action of metformin in survival signaling was mediated by the AMPK pathway.

To better understand the profile of metformin between several signal pathways in neuroprotection, we assayed genes and proteins whose expressions or activities are either directly or indirectly affected by the AMPK pathway. It is different that AMPK can act through various roles of several cell type specific functions (e.g., mitochondrial biogenesis, cellular synthetic function, anti-inflammation, anti-oxidative stress, cell growth and proliferation) and molecular mechanisms (e.g., integration of proper effects via AMPK–PPAR γ , AMPK–PGC1 α , AMPK–PFK, AMPK–FOXO, and AMPK–mTOR signaling cascades) [30,38,52,75,76]. Nevertheless, our identification of the AMPK and its downstream genes will help to explain the mechanisms by which neuroprotective actions are regulated in response to survivability and mitochondrial function in the hNSCs with AGEs. Furthermore, our discovery that the AMPK converges with its downstream gene pathway at several actions, associated with the reciprocal effects of metformin and Compound C on AMPK activity and expression, implies that a mediatory relationship role and precise mechanisms will require further investigation in diabetic central neuropathy.

In the study, we showed that metformin activates AMPK to rescue the AGE-mediated neurotoxic effects in hNSCs. Our study clarifies AMPK in hNSCs as a critical target of AGE-mediated neurodegenerative pathological effects, and shows that these effects can be rescued by up-regulating AMPK expression and activity. This suggests that AMPK is a potential therapeutic drug target worthy of further assessment in diabetic and other neurodegenerative diseases among patients affected by these diseases.

Fig. 11. Metformin enhanced the expression of neuroprotective genes in the hNSCs treated with AGEs. hNSCs were treated with AGEs (0.5 mg/ml) for 24 h, then treated with the indicated reagents (1 mM metformin or 10 μ M Compound C) for another 48 h. The PPAR γ (A), Bcl-2 (B) and CREB (C) transcripts in the indicated hNSCs were analyzed using the Q-PCR technique. RNA of the indicated hNSCs was collected and reverse-transcribed into cDNA. Q-PCR technique of the indicated gene was performed and normalized to that of GAPDH. All reactions were run in triplicate from each independent experiment. Values A, B and C are expressed as a percentage of the indicated transcript in CON and are presented as the mean \pm SEM values from three independent experiments. ^a Specific comparison to the indicated hNSCs with AGEs ($p < 0.001$; one-way ANOVA).

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

We thank Chia-Nan Yen for proof-reading and editing the manuscript. This work was supported by grants from the Cardinal Tien Hospital (CTH-103-1-2B06 to M.M.C and M.C.C.), Ministry of Science and Technology (MOST 103-2320-B-030-006 to M.C.C.), Major State Basic Research Program of China (2014CB964602 to B.S.), the National Natural Science Foundation of China (91132713 to B.S.), Fu Jen Catholic University, and Terry Whole Brain & Potential Development Center. As I constructed this manuscript, my beloved father, Ying-Hsiung Chiang, just had an operation. I had accompanied him to take chemical therapy at National Taiwan University Hospital Yun-Lin Branch for three months. Now the manuscript is completed, I would like to dedicate this work to my late father.

References

- [1] D.A. Butterfield, F. Di Domenico, E. Barone, Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain, *Biochim. Biophys. Acta* 1842 (2014) 1693–1706.
- [2] A.L. McCall, The impact of diabetes on the CNS, *Diabetes* 41 (1992) 557–570.
- [3] V.S. Thomas, S. Darvesh, C. MacKnight, K. Rockwood, Estimating the prevalence of dementia in elderly people: a comparison of the Canadian study of health and aging and national population health survey approaches, *Int. Psychogeriatr.* 13 (Suppl. 1) (2001) 169–175.
- [4] S.E. Arnold, I. Lucki, B.R. Brookshire, G.C. Carlson, C.A. Browne, H. Kazi, S. Bang, B.R. Choi, Y. Chen, M.F. McMullen, S.F. Kim, High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice, *Neurobiol. Dis.* 67 (2014) 79–87.
- [5] A. Ott, R.P. Stolk, F. van Harskamp, H.A. Pols, A. Hofman, M.M. Breteler, Diabetes mellitus and the risk of dementia: the Rotterdam study, *Neurology* 53 (1999) 1937–1942.
- [6] R. Peila, B.L. Rodriguez, L.J. Launer, Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: the Honolulu–Asia aging study, *Diabetes* 51 (2002) 1256–1262.
- [7] Z. Arvanitakis, R.S. Wilson, J.L. Bienias, D.A. Evans, D.A. Bennett, Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function, *Arch. Neurol.* 61 (2004) 661–666.
- [8] T. den Heijer, S.E. Vermeer, E.J. van Dijk, N.D. Prins, P.J. Koudstaal, A. Hofman, M.M. Breteler, Type 2 diabetes and atrophy of medial temporal lobe structures on brain MRI, *Diabetologia* 46 (2003) 1604–1610.
- [9] R. Stewart, D. Liolitsa, Type 2 diabetes mellitus, cognitive impairment and dementia, *Diabet. Med.* 16 (1999) 93–112.
- [10] P.J. Magistretti, L. Pellerin, Cellular mechanisms of brain energy metabolism. Relevance to functional brain imaging and to neurodegenerative disorders, *Ann. N. Y. Acad. Sci.* 777 (1996) 380–387.
- [11] A.I. Duarte, E. Candeias, S.C. Correia, R.X. Santos, C. Carvalho, S. Cardoso, A. Placido, M.S. Santos, C.R. Oliveira, P.I. Moreira, Crosstalk between diabetes and brain: glucagon-like peptide-1 mimetics as a promising therapy against neurodegeneration, *Biochim. Biophys. Acta* 1832 (2013) 527–541.
- [12] P.T. Nelson, C.D. Smith, E.A. Abner, F.A. Schmitt, S.W. Scheff, G.J. Davis, J.N. Keller, G.A. Jicha, D. Davis, W. Wang-Xia, A. Hartman, D.G. Katz, W.R. Markesbery, Human cerebral neuropathology of type 2 diabetes mellitus, *Biochim. Biophys. Acta* 1792 (2009) 454–469.
- [13] S. Yamagishi, M. Takeuchi, Y. Inagaki, K. Nakamura, T. Imaizumi, Role of advanced glycation end products (AGEs) and their receptor (RAGE) in the pathogenesis of diabetic microangiopathy, *Int. J. Clin. Pharmacol. Res.* 23 (2003) 129–134.
- [14] S. Yamagishi, T. Imaizumi, Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy, *Curr. Pharm. Des.* 11 (2005) 2279–2299.
- [15] S.H. Wang, Z.L. Sun, Y.J. Guo, Y. Yuan, L. Li, PPARgamma-mediated advanced glycation end products regulation of neural stem cells, *Mol. Cell. Endocrinol.* 307 (2009) 176–184.
- [16] S. Yamagishi, K. Nakamura, T. Matsui, S. Ueda, K. Fukami, S. Okuda, Agents that block advanced glycation end product (AGE)-RAGE (receptor for AGEs)-oxidative stress system: a novel therapeutic strategy for diabetic vascular complications, *Expert Opin. Investig. Drugs* 17 (2008) 983–996.
- [17] D. Ruggiero-Lopez, M. Lecomte, G. Moinet, G. Patereau, M. Lagarde, N. Wiernsperger, Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation, *Biochem. Pharmacol.* 58 (1999) 1765–1773.
- [18] G.R. Steinberg, B.E. Kemp, AMPK in health and disease, *Physiol. Rev.* 89 (2009) 1025–1078.
- [19] S. Fogarty, D.G. Hardie, Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer, *Biochim. Biophys. Acta* 1804 (2010) 581–591.
- [20] W. Sun, T.S. Lee, M. Zhu, C. Gu, Y. Wang, Y. Zhu, J.Y. Shyy, Statins activate AMP-activated protein kinase in vitro and in vivo, *Circulation* 114 (2006) 2655–2662.
- [21] S. Rahbar, R. Natarajan, K. Yermeni, S. Scott, N. Gonzales, J.L. Nadler, Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation, *Clin. Chim. Acta* 301 (2000) 65–77.
- [22] G.V. Ronnett, S. Ramamurthy, A.M. Kleman, L.E. Landree, S. Aja, AMPK in the brain: its roles in energy balance and neuroprotection, *J. Neurochem.* 109 (Suppl. 1) (2009) 17–23.
- [23] D. Carling, The AMP-activated protein kinase cascade—a unifying system for energy control, *Trends Biochem. Sci.* 29 (2004) 18–24.
- [24] D.G. Hardie, Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status, *Endocrinology* 144 (2003) 5179–5183.
- [25] S.M. Shin, I.J. Cho, S.G. Kim, Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycogen synthase kinase-3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway, *Mol. Pharmacol.* 76 (2009) 884–895.
- [26] S.L. Choi, S.J. Kim, K.T. Lee, J. Kim, J. Mu, M.J. Birnbaum, S. Soo Kim, J. Ha, The regulation of AMP-activated protein kinase by H(2)O(2), *Biochem. Biophys. Res. Commun.* 287 (2001) 92–97.
- [27] I.P. Salt, G. Johnson, S.J. Ashcroft, D.G. Hardie, AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release, *Biochem. J.* 335 (Pt 3) (1998) 533–539.
- [28] K. Arsikin, T. Kravic-Stevovic, M. Jovanovic, B. Ristic, G. Tovilovic, N. Zogovic, V. Bumbasirevic, V. Trajkovic, L. Harhaji-Trajkovic, Autophagy-dependent and -independent involvement of AMP-activated protein kinase in 6-hydroxydopamine toxicity to SH-SY5Y neuroblastoma cells, *Biochim. Biophys. Acta* 1822 (2012) 1826–1836.
- [29] O.K. Melemedjian, M.N. Asiedu, D.V. Tillu, R. Sanoja, J. Yan, A. Lark, A. Khoutorsky, J. Johnson, K.A. Peebles, T. Lepow, N. Sonenberg, G. Dussor, T.J. Price, Targeting adenosine monophosphate-activated protein kinase (AMPK) in preclinical models reveals a potential mechanism for the treatment of neuropathic pain, *Mol. Pain* 7 (2011) 70.
- [30] B.B. Zhang, G. Zhou, C. Li, AMPK: an emerging drug target for diabetes and the metabolic syndrome, *Cell Metab.* 9 (2009) 407–416.
- [31] A. Salminen, K. Kaarniranta, AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network, *Ageing Res. Rev.* 11 (2012) 230–241.
- [32] A. Salminen, K. Kaarniranta, A. Haapasalo, H. Soininen, M. Hiltunen, AMP-activated protein kinase: a potential player in Alzheimer's disease, *J. Neurochem.* 118 (2011) 460–474.
- [33] M. Dulovic, M. Jovanovic, M. Xilouri, L. Stefanis, L. Harhaji-Trajkovic, T. Kravic-Stevovic, V. Paunovic, M.T. Ardah, O.M. El-Agnaf, V. Kostic, I. Markovic, V. Trajkovic, The protective role of AMP-activated protein kinase in alpha-synuclein neurotoxicity in vitro, *Neurobiol. Dis.* 63 (2014) 1–11.
- [34] A. Ferretta, A. Gaballo, P. Tanzarella, C. Piccoli, N. Capitanio, B. Nico, T. Annesse, M. Di Paola, C. Dell'acqua, M. De Mari, E. Ferrarini, V. Bonifati, C. Pacelli, T. Cocco, Effect of resveratrol on mitochondrial function: implications in parkin-associated familial Parkinson's disease, *Biochim. Biophys. Acta* 1842 (2014) 902–915.
- [35] A. Chakraborty, S. Chowdhury, M. Bhattacharyya, Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients, *Diabetes Res. Clin. Pract.* 93 (2011) 56–62.
- [36] A. Salminen, J.M. Hyttinen, K. Kaarniranta, AMP-activated protein kinase inhibits NF-kappaB signaling and inflammation: impact on healthspan and lifespan, *J. Mol. Med. (Berl.)* 89 (2011) 667–676.
- [37] M.C. Chiang, H. Lin, Y.C. Cheng, C.H. Yen, R.N. Huang, K.H. Lin, Beta-adrenoceptor pathway enhances mitochondrial function in human neural stem cells via rotary cell culture system, *J. Neurosci. Methods* 207 (2012) 130–136.
- [38] M.C. Chiang, Y.C. Cheng, H.M. Chen, Y.J. Liang, C.H. Yen, Rosiglitazone promotes neurite outgrowth and mitochondrial function in N2A cells via PPARgamma pathway, *Mitochondrion* 14 (2014) 7–17.
- [39] M.C. Chiang, Y.C. Cheng, K.H. Lin, C.H. Yen, PPARgamma regulates the mitochondrial dysfunction in human neural stem cells with tumor necrosis factor alpha, *Neuroscience* 229 (2013) 118–129.
- [40] M.C. Chiang, Y. Chern, R.N. Huang, PPARgamma rescue of the mitochondrial dysfunction in Huntington's disease, *Neurobiol. Dis.* 45 (2012) 322–328.
- [41] M.C. Chiang, Y. Chern, C.G. Juo, The dysfunction of hepatic transcriptional factors in mice with Huntington's disease, *Biochim. Biophys. Acta* 1812 (2011) 1111–1120.
- [42] R. Ramasamy, S.J. Vannucci, S.S. Yan, K. Herold, S.F. Yan, A.M. Schmidt, Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation, *Glycobiology* 15 (2005) 16R–28R.
- [43] V. Srikanth, A. Maczurek, T. Phan, M. Steele, B. Westcott, D. Juskiw, G. Munch, Advanced glycation endproducts and their receptor RAGE in Alzheimer's disease, *Neurobiol. Aging* 32 (2011) 763–777.
- [44] S.F. Yan, S.D. Yan, R. Ramasamy, A.M. Schmidt, Tempering the wrath of RAGE: an emerging therapeutic strategy against diabetic complications, neurodegeneration, and inflammation, *Ann. Med.* 41 (2009) 408–422.
- [45] Y. Ishibashi, S. Yamagishi, T. Matsui, K. Ohta, R. Tanoue, M. Takeuchi, S. Ueda, K. Nakamura, S. Okuda, Pravastatin inhibits advanced glycation end products (AGEs)-induced proximal tubular cell apoptosis and injury by reducing receptor for AGEs (RAGE) level, *Metab. Clin. Exp.* 61 (2012) 1067–1072.
- [46] J. Gasic-Milenkovic, C. Loske, G. Munch, Advanced glycation endproducts cause lipid peroxidation in the human neuronal cell line SH-SY5Y, *J. Alzheimers Dis.* 5 (2003) 25–30.

- [47] P.C. Chang, L.Y. Chong, S.C. Tsai, L.P. Lim, Aminoguanidine inhibits the AGE-RAGE axis to modulate the induction of periodontitis but has limited effects on the progression and recovery of experimental periodontitis: a preliminary study, *J. Periodontol.* 85 (2014) 729–739.
- [48] P.J. Thornalley, Use of aminoguanidine (pimagedine) to prevent the formation of advanced glycation endproducts, *Arch. Biochem. Biophys.* 419 (2003) 31–40.
- [49] D.R. Green, J.C. Reed, Mitochondria and apoptosis, *Science* 281 (1998) 1309–1312.
- [50] M.I. Naseer, I. Ullah, M.L. Narasimhan, H.Y. Lee, R.A. Bressan, G.H. Yoon, D.J. Yun, M.O. Kim, Neuroprotective effect of osmotin against ethanol-induced apoptotic neurodegeneration in the developing rat brain, *Cell Death Dis.* 5 (2014) e1150.
- [51] M. Saito, G. Chakraborty, R.F. Mao, R. Wang, T.B. Cooper, C. Vadasz, Ethanol alters lipid profiles and phosphorylation status of AMP-activated protein kinase in the neonatal mouse brain, *J. Neurochem.* 103 (2007) 1208–1218.
- [52] N.A. Shirwany, M.H. Zou, AMPK: a cellular metabolic and redox sensor. A minireview, *Landmark ed., Front Biosci.*, 19, 2014, pp. 447–474.
- [53] R.C. Scarpulla, Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network, *Biochim. Biophys. Acta* 1813 (2011) 1269–1278.
- [54] S. Jager, C. Handschin, J. St-Pierre, B.M. Spiegelman, AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 α , *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 12017–12022.
- [55] P. Puigserver, B.M. Spiegelman, Peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α): transcriptional coactivator and metabolic regulator, *Endocr. Rev.* 24 (2003) 78–90.
- [56] Z. Wu, P. Puigserver, U. Andersson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, R.C. Scarpulla, B.M. Spiegelman, Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1, *Cell* 98 (1999) 115–124.
- [57] I. Pagel-Langenickel, J. Bao, J.J. Joseph, D.R. Schwartz, B.S. Mantell, X. Xu, N. Raghavachari, M.N. Sack, PGC-1 α integrates insulin signaling, mitochondrial regulation, and bioenergetic function in skeletal muscle, *J. Biol. Chem.* 283 (2008) 22464–22472.
- [58] L.M. Jimenez-Flores, S. Lopez-Briones, M.H. Macias-Cervantes, J. Ramirez-Emiliano, V. Perez-Vazquez, A PPAR γ , NF- κ B and AMPK-dependent mechanism may be involved in the beneficial effects of curcumin in the diabetic db/db mice liver, *Molecules* 19 (2014) 8289–8302.
- [59] F. Alrashed, D. Calay, C. Thornton, A. Bauer, A. Kiprianos, D. Haskard, J. Boyle, J. Mason, P179Celecoxib-mediated activation of an AMPK-CREB-Nrf2 dependent pathway: a novel mechanism for endothelial cytoprotection in chronic systemic inflammation, *Cardiovasc. Res.* 103 (Suppl. 1) (2014) S31–S32.
- [60] J.N. Xu, C. Zeng, Y. Zhou, C. Peng, Y.F. Zhou, Q. Xue, Metformin inhibits StAR expression in human endometrial stromal cells via AMPK-mediated disruption of CREB-CRTC2 complex formation, *J. Clin. Endocrinol. Metab.* 99 (2014) 2795–2803.
- [61] S.S. Hamdulay, B. Wang, D. Calay, A.P. Kiprianos, J. Cole, O. Dumont, N. Dryden, A.M. Randi, C.C. Thornton, F. Al-Rashed, C. Hoong, A. Shamsi, Z. Liu, V.R. Holla, J.J. Boyle, D.O. Haskard, J.C. Mason, Synergistic therapeutic vascular cytoprotection against complement-mediated injury induced via a PKC α -AMPK-, and CREB-dependent pathway, *J. Immunol.* 192 (2014) 4316–4327.
- [62] C. Moran, A. Sanz-Rodriguez, A. Jimenez-Pacheco, J. Martinez-Villareal, R.C. McKiernan, E.M. Jimenez-Mateos, C. Mooney, I. Woods, J.H. Prehn, D.C. Henshall, T. Engel, Bmf upregulation through the AMP-activated protein kinase pathway may protect the brain from seizure-induced cell death, *Cell Death Dis.* 4 (2013) e606.
- [63] B. Kuhla, C. Loske, S. Garcia De Arriba, R. Schinzel, J. Huber, G. Munch, Differential effects of “advanced glycation endproducts” and beta-amyloid peptide on glucose utilization and ATP levels in the neuronal cell line SH-SY5Y, *J. Neural Transm.* 111 (2004) 427–439.
- [64] S.G. de Arriba, G. Stuchbury, J. Yarin, J. Burnell, C. Loske, G. Munch, Methylglyoxal impairs glucose metabolism and leads to energy depletion in neuronal cells—protection by carbonyl scavengers, *Neurobiol. Aging* 28 (2007) 1044–1050.
- [65] P. Wareski, A. Vaarmann, V. Choubey, D. Safulina, J. Liiv, M. Kuum, A. Kaasik, PGC-1 { α } and PGC-1{ β } regulate mitochondrial density in neurons, *J. Biol. Chem.* 284 (2009) 21379–21385.
- [66] A. Kamal, G.J. Biessels, S.E. Duis, W.H. Gispen, Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: interaction of diabetes and ageing, *Diabetologia* 43 (2000) 500–506.
- [67] I. Ullah, N. Ullah, M.I. Naseer, H.Y. Lee, M.O. Kim, Neuroprotection with metformin and thymoquinone against ethanol-induced apoptotic neurodegeneration in prenatal rat cortical neurons, *BMC Neurosci.* 13 (2012) 11.
- [68] M.Y. El-Mir, D. Detaille, R.V.G., M. Delgado-Esteban, B. Guigas, S. Attia, E. Fontaine, A. Almeida, X. Lerveur, Journal of molecular neuroscience, Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons, *J. Mol. Neurosci.* 34 (2008) 77–87 MN.
- [69] R.M. Reznick, H. Zong, J. Li, K. Morino, I.K. Moore, H.J. Yu, Z.X. Liu, J. Dong, K.J. Mustard, S.A. Hawley, D. Befroy, M. Pypaert, D.G. Hardie, L.H. Young, G.I. Shulman, Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis, *Cell Metab.* 5 (2007) 151–156.
- [70] N.L. Price, A.P. Gomes, A.J. Ling, F.V. Duarte, A. Martin-Montalvo, B.J. North, B. Agarwal, L. Ye, G. Ramadori, J.S. Teodoro, B.P. Hubbard, A.T. Varela, J.G. Davis, B. Varamini, A. Hafner, R. Moaddel, A.P. Rolo, R. Coppari, C.M. Palmeira, R. de Cabo, J.A. Baur, D.A. Sinclair, SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function, *Cell Metab.* 15 (2012) 675–690.
- [71] S.K. Chowdhury, D.R. Smith, P. Fernyhough, The role of aberrant mitochondrial bioenergetics in diabetic neuropathy, *Neurobiol. Dis.* 51 (2013) 56–65.
- [72] J. Choi, K. Chandrasekaran, T. Inoue, A. Muragundla, J.W. Russell, PGC-1 α regulation of mitochondrial degeneration in experimental diabetic neuropathy, *Neurobiol. Dis.* 64 (2014) 118–130.
- [73] T.L. Horvath, D.M. Erion, J.D. Elsworth, R.H. Roth, G.I. Shulman, Z.B. Andrews, GPA protects the nigrostriatal dopamine system by enhancing mitochondrial function, *Neurobiol. Dis.* 43 (2011) 152–162.
- [74] M. Suwa, T. Egashira, H. Nakano, H. Sasaki, S. Kumagai, Metformin increases the PGC-1 α protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo, *J. Appl. Physiol.* 101 (2006) 1685–1692.
- [75] D.G. Hardie, AMPK: positive and negative regulation, and its role in whole-body energy homeostasis, *Curr. Opin. Cell Biol.* 33C (2014) 1–7.
- [76] J.A. Godoy, J.A. Rios, J.M. Zolezzi, N. Braidy, N.C. Inestrosa, Signaling pathway cross talk in Alzheimer's disease, *Cell Commun. Signal.* 12 (2014) 23.