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Adiponectin is Protective against Oxidative Stress Induced Cytotoxicity in Amyloid-Beta Neurotoxicity

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Abstract

Beta-amyloid (Aβ) neurotoxicity is important in Alzheimer's disease (AD) pathogenesis. Aβ neurotoxicity causes oxidative stress, inflammation and mitochondrial damage resulting in neuronal degeneration and death. Oxidative stress, inflammation and mitochondrial failure are also pathophysiological mechanisms of type 2 diabetes (T2DM) which is characterized by insulin resistance. Interestingly, T2DM increases risk to develop AD which is associated with reduced neuronal insulin sensitivity (central insulin resistance). We studied the potential protective effect of adiponectin (an adipokine with insulin-sensitizing, anti-inflammatory and anti-oxidant properties) against Aβ neurotoxicity in human neuroblastoma cells (SH-SY5Y) transfected with the Swedish amyloid precursor protein (Sw-APP) mutant, which overproduced Aβ with abnormal intracellular Aβ accumulation. Cytotoxicity was measured by assay for lactate dehydrogenase (LDH) released upon cell death and lysis. Our results revealed that Sw-APP transfected SH-SY5Y cells expressed both adiponectin receptor 1 and 2, and had increased AMP-activated protein kinase (AMPK) activation and enhanced nuclear factor-kappa B (NF-xB) activation compared to control empty-vector transfected SH-SY5Y cells. Importantly, adiponectin at physiologic concentration of 10 μg/ml protected Sw-APP transfected SH-SY5Y cells against cytotoxicity under oxidative stress induced by hydrogen peroxide. This neuroprotective action of adiponectin against Aβ neurotoxicity-induced cytotoxicity under oxidative stress involved 1) AMPK activation mediated via the endosomal adaptor protein APPL1 (adaptor protein with phosphotyrosine binding, pleckstrin homology domains and leucine zippper motif) and possibly 2) suppression of NF-xB activation. This raises the possibility of novel therapies for AD such as adiponectin receptor agonists.

Introduction

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly with significant morbidity and mortality [1]. The exact pathogenetic mechanisms underlying AD are uncertain. One extensively studied mechanism is neurotoxicity mediated by beta-amyloid (Aβ) [2–5]. Histopathological studies of brain from AD patient reveal extracellular accumulation of senile plaques containing Aβ fibrils, intracellular accumulation of neurofibrillary tangles containing hyperphosphorylated tau, neuronal loss, amyloid angiopathy, and inflammation [1,5–6]. Aβ peptides, predominantly Aβ40 and Aβ42, are derived from cleavage of amyloid precursor protein (APP), by β secretase and γ secretase [2]. Aβ exist in different forms including monomers (peptides), oligomers, protofibrils and fibrils [7]. The pathogenetic role of Aβ in AD is strongly supported by the observation that familial AD patients have mutations affecting proteins involved in Aβ production or processing such as APP, presenilin1 and presenilin 2. An example is the Swedish APP mutation (Sw-APP, APPK670N, M671L ) that causes familial early-onset AD [8]. Aβ is neurotoxic [2–4]. Recent evidences suggest that Aβ oligomers are directly toxic to neurons and play important roles in early AD [9–12]. Aβ oligomers inhibit long-term potentiation in hippocampal neurons [12], impair neuronal synaptic transmission by causing loss of excitatory synapses and dendritic spines [13–14], and may induce uncontrolled ion flux by forming Ca2+-permeable pores in the lipid membrane [15–16].

Type 2 diabetes mellitus (T2DM) is, similar to AD, common in the elderly with significant morbidity and mortality. Interestingly, several pathophysiological features of T2DM are also found in AD. These include 1) insulin resistance, 2) inflammation, 3) oxidative stress, and 4) aberrant lipid metabolism [17]. In AD, there are 1) central insulin resistance resulting from reduction of insulin receptors and desensitization of insulin receptors in neurons [18–
Adiponectin Protective against Abeta Neurotoxicity

Site-directed Mutagenesis of the Sw-APP

Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using the QuickChange Lightning Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol. The mutagenic primers used for generating the Sw-APP-mutated construct were: forward GGAGATCTCTGAGTGAACTTGAGTGGATGCGAACATTCCGAC, and reverse GTCCGAAATTCGATCATCAGGTCCACGATGCTG.

Reverse Transcriptase Polymerase Chain Reaction for Expression of Adiponectin Receptors

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to study the expression of adiponectin receptors, AdipoR1 and AdipoR2, by SH-SY5Y cells. The primers used for human AdipoR1 are: forward GAGCATGCTTCCGGCATTCTA and reverse AAGGCGCAGGAGAAGCTGAG. The primers used for human AdipoR2 are: forward GACCTTTCTCCTTGCCATGGA and reverse AAAGGAGATATTTGGGCGAA.

Enzyme Linked Immunosorbent Assay (ELISA) for Abeta Oligomers

Concentrations of secreted Abeta oligomers in culture medium of the three groups of cells were measured using ELISA recently reported by our group [43] and compared using ANOVA. In brief, the ELISA employed rabbit antibody against Abeta N-terminal (Abeta residues 1–14) (Abcam) as the capturing antibody and the Abeta oligomers antibody, 7A1a (New England Agent), as the detecting antibody.

Measurement of Cytotoxicity under Oxidative Stress and Neuroprotection by Adiponectin

Cell death and lysis (cytotoxicity) was assessed by examining under light microscope and measured by assay of lactate dehydrogenase (LDH) level. Cells were examined under light microscope at 10X and 100X for cell morphology with loss of dendritic spines (ballooning) as features suggestive of cell death. LDH release into medium was quantitated by cytotoxicity detection kit (Roche Applied Science, Germany) to assess cytotoxicity of the three groups of SH-SY5Y cells: 1) cells transfected with empty-vector, 2) cells transfected with wt-APP and 3) cells transfected with Sw-APP mutant before and after exposure to oxidative stress. Oxidative stress was induced with addition of H2O2 to culture medium at 200 μM, 400 μM and 800 μM for 2 hours. Sw-APP transfected SH-SY5Y cells (2×10⁵)

Methods

The study was approved by the Hong Kong University-Hong Kong West Cluster Institutional Review Board. Human neuroblastoma cells (SH-SY5Y cell line) were transfected with the wild-type human APP (wt-APP) or Swedish-APP mutant (Sw-APP), and control being SH-SY5Y cells transfected with empty-vector. Cultured SH-SY5Y cells transfected with Sw-APP mutant overexpress APP with increased Abeta production by 5-6 fold, concomitant increase in secreted Abeta and abnormal intracellular Abeta accumulation [39–41]. Cells transfected with wt-APP overexpress APP with increased Abeta production, but without abnormal intracellular Abeta accumulation [42]. The processing pathways for secreted Abeta and intracellular Abeta are different [41,43]. SH-SY5Y cells transfected with Sw-APP mutant were used as an in-vitro system for further study. PC-12 (rat pheochromocytoma) cells transfected with Sw-APP mutant were shown to have increased Abeta production and enhanced vulnerability to oxidative stress-induced apoptosis [44].

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Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using the QuickChange Lightning Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol. The mutagenic primers used for generating the Sw-APP-mutated construct were: forward GGAGATCTCTGAGTGAACTTGAGTGGATGCGAACATTCCGAC, and reverse GTCCGAAATTCGATCATCAGGTCCACGATGCTG.

Cell Culture and Transfection

SH-SY5Y cell line was obtained from ATCC and maintained at 37°C (5% CO2/95% air) in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen, USA) and 1% penicillin and streptomycin. SH-SY5Y cells were transfected with empty-vector, vector carrying wt-APP and Sw-APP mutant under standard protocols using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s instructions. Transfection was confirmed with DNA sequencing and stably transfected cells were selected with G418. Expression of the Sw-APP mutant with abnormal increase of intracellular accumulation of Abeta was confirmed with western blot of cell lysates using rabbit antibody against amino acids 1–16 of the amino terminal of human Abeta (Santa Cruz, CA).

Reverse Transcriptase Polymerase Chain Reaction for Expression of Adiponectin Receptors

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to study the expression of adiponectin receptors, AdipoR1 and AdipoR2, by SH-SY5Y cells. The primers used for human AdipoR1 are: forward GAGCATGCTTCCGGCATTCTA and reverse AAGGCGCAGGAGAAGCTGAG. The primers used for human AdipoR2 are: forward GACCTTTCTCCTTGCCATGGA and reverse AAAGGAGATATTTGGGCGAA.

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were seeded on each well of a 24-well plate one day before use, each well contained 500 μl of DMEM containing 10% FBS (Invitrogen, USA) and 1% penicillin and streptomycin. Two hours before addition of H2O2, fresh culture medium without FBS was replaced and LDH level was measured 2 hours after addition of H2O2 according to manufacturer’s instructions. Potential neuroprotective effect of adiponectin was studied using Sw-APP transfected SH-SY5Y cells by adding adiponectin to fresh culture medium without FBS at 10 μg/ml for 2 hours before addition of H2O2. Mouse recombinant adiponectin was produced and purified as described previously [46]. LDH levels were used to calculate percentage cytotoxicity which was expressed as fold of cytotoxicity compared to percentage cytotoxicity of cells without adiponectin treatment or exposure to H2O2. Folds of cytotoxicity was compared between cells without adiponectin treatment before exposure to H2O2 and cells pretreated with adiponectin before exposure to H2O2.

**Immunoblotting**

Culture medium was aspirated from wells of 24-well plate. Cells were washed with ice-cold 1xPBS and removed. 100 μl of 1x lysis buffer (Cell Signaling Technology, USA) was added to each well and incubated on ice for 20 minutes with gentle shaking to allow complete lysis. The cell lysate was transferred to a 1.5 ml centrifuge tube and centrifuged at 14,000 rpm at 4°C for 10 minutes. After centrifugation, the supernatant was transferred to a new 1.5 ml centrifuge tube for Western blot analysis. Lysates of cultured cells were assessed for Aβ oligomers, by standard SDS-PAGE electrophoresis and immunoblotting using an Aβ oligomer antibody, 7A1a, as described recently [45]. In addition, expression levels of APP1, total AMPK and pAMPK were studied by standard SDS-PAGE and immunoblotting using anti-APP1 antibody and anti-pAMPK antibody as primary antibodies. Anti-human APP1 antibody was synthesized by immunization of New Zealand female rabbits with the recombinant full-length APP1 produced from Escherichia Coli using protocol described previously [47]. Antibody against AMPKα subunit phosphorylated at threonine172 (anti-pAMPK Thr172) was purchased from Cell Signaling (Beverly, MA, USA). The specific signals were amplified by addition of horseradish peroxidase-conjugated antibodies and visualized using an enhanced chemiluminescence (Amersham, UK).

**Knockdown of AdipoR1, AdipoR2 and APPL1 Expression by siRNA**

Knockdown of AdipoR1 and AdipoR2 expression in Sw-APP transfected SH-SY5Y cells was performed using RNA interference by transfection with duplex stealth interference RNA (siRNA) for adipor1 and adipor2 (Invitrogen, USA) or scrambled RNA (negative control) using Lipofectamine 2000 according to manufacturer’s instructions as previously described by our group [47]. Knockdown of APPL1 expression in Sw-APP transfected SH-SY5Y cells was achieved similarly by siRNA for APPL1 (Invitrogen, USA) or scrambled RNA (negative control). Successful knockdown of AdipoR1, AdipoR2 and APPL1 expression was confirmed by western blot of cell lysates. Cells were then pretreated with adiponectin at 10 μg/ml for 2 hours before addition of H2O2 (400 μM) followed by cytotoxicity assay 2 hours later to study whether the neuroprotective effect of adiponectin required expression of AdipoR1, AdipoR2 and APPL1.

**Western Blot for NF-κB in Nuclear Fraction of SH-SY5Y Cells**

Levels of NF-κB activation of the three groups of cells, empty-vector transfected, wt-APP transfected and Sw-APP transfected SH-SY5Y cells were studied by western blot for NF-κB p65 in nuclear fraction of the cells. Nuclear extracts of the SH-SY5Y cells were prepared according to the standard procedures of a Nuclear Extraction Kit (Panomics, Inc.; Beijing, China). Protein concentration of the nuclear extracts was measured, followed by standard procedures of protein transfer of western blot. The nitrocellulose membrane was incubated with NF-κB p65 antibody (Santa Cruz, California; dilution 1:1,000) or histone H3 antibody (Abcam, Inc.,
U.K.; dilution 1:1,000) as primary antibody at 4°C for overnight. After washing with 1X TBST buffer 3 times (5 min each), the membranes were incubated with anti-rabbit secondary antibody (DAKO, Denmark; dilution 1:5,000) at RT for one hour and then washed again with 1X TBST buffer 3 times. The membranes were then immersed in ECL detection solution (Amersham Biosciences) for 1 min in darkness. Excess detection solution was drained off and the membrane was then wrapped in transparent wrapping film and exposed to X-ray film for 2 min. Images of the X-ray film were analyzed with Image-J. The effect of adiponectin on NF-kB activation of Sw-APP transfected SH-SY5Y cells was further studied. Sw-APP transfected SH-SY5Y cells were cultured in 6-well plate at one million cells per well one night before the experiment. Sw-APP transfected SH-SY5Y cells were divided into 4 groups: control cells without pretreatment with adiponectin (ADN) or exposure to H2O2 (group 1), cells treated with ADN (10 μg/ml) for 2 hours without exposure to H2O2 (group 2), cells exposed to 400 μM H2O2 for 2 hours without ADN pretreatment before exposure to H2O2 (group 3), and cells pretreated with ADN for 2 hours before exposure to 400 μM H2O2 for 2 hours (group 4). After treatment, nuclear extracts of the cells were prepared and western blot for NF-kB p65 was performed as described above. The amount of NF-kB p65 was quantitated by Image-J analysis.

Statistical Analysis

Western blot results of different groups of cells were quantified by Image-J and compared by one-way ANOVA using Newman-Keuls multiple comparison test with p-value <0.05 considered as statistically significant. All ELISA and cytotoxicity experiments were performed at least 3 times and the mean values of results from different groups of cells were similarly compared by one-way ANOVA using Newman-Keuls multiple comparison test with p-value <0.05 considered as statistically significant.

Results

Human Neuroblastoma Cells Express both Adiponectin Receptor 1 and 2

RT-PCR results demonstrated that SH-SY5Y cells express both AdipoR1 and AdipoR2 (Figure 1). A previous study has demonstrated that SH-SY5Y cells express AdipoR2 by western blot analysis [48].

Figure 2. Sw-APP transfected SH-SY5Y cells secrete Aβ oligomers and have increased intracellular Aβ oligomers level.

A. Western blot analysis of cell lysates with Aβ antibody. Immunoblotting demonstrated that SH-SY5Y cells transfected with Swedish amyloid precursor protein (Sw-APP) mutant had increased amount of intracellular Aβ oligomers (tetramers and hexamers) compared to SH-SY5Y cells transfected with wild-type amyloid precursor protein (APP), empty-vector (vector) or untransfected SH-SY5Y cells (UT). B. Image J quantitative analysis confirmed that Sw-APP transfected cells (Sw-APP) had increased amount of intracellular hexamers compared to empty-vector transfected (vector) (p<0.05) or untransfected (UT) cells (p<0.05).

B. ELISA for concentration of secreted Aβ oligomers in cultured medium. Sw-APP transfected SH-SY5Y cells (Sw-APP) secreted greater amount of extracellular Aβ oligomers than wt-APP transfected cells (APP) (p=0.0001), which also secreted greater amount of extracellular Aβ oligomers than empty-vector transfected cells (vector) (p<0.05). Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test.

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Figure 3. Western blot analysis of cell lysates for phosphorylated AMP-activated protein kinase (pAMPK) and the endosomal adaptor protein APPL-1 (adaptor protein with phosphotyrosine binding, pleckstrin homology domains and leucine zipper motif). Immunoblotting demonstrated that SH-SY5Y cells transfected with Sw-APP mutant (Sw-APP) had increased pAMPK than SH-SY5Y cells transfected with wt-APP (APP) which had increased pAMPK than control cells transfected with empty-vector (vector). Expression of APPL1 was indifferent among the three groups of cells. Experiments were repeated 3 times with consistent results.

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Adiponectin Protective against Abeta Neurotoxicity

Human Neuroblastoma Cells Stably Transfected with Sw-APP Mutant were more Vulnerable to Cytotoxicity Under Oxidative Stress

Without addition of H₂O₂, the basal cytotoxicity/death rates of Sw-APP transfected, wt-APP transfected and empty-vector transfected SH-SY5Y cells were 0.807%, 0.863% and 0.848% respectively, being indifferent (Figure 4A). Upon exposure to H₂O₂ induced oxidative stress, Sw-APP transfected SH-SY5Y cells had higher cytotoxicity compared to wt-APP transfected cells (p<0.0001) which had higher cytotoxicity compared to empty-vector transfected cells (p<0.0001) (Figure 4B). This supports that increased Aβ production particularly with abnormal intracellular Aβ accumulation enhances vulnerability to cytotoxicity with cell lysis under oxidative stress [45].

Adiponectin was Protective against Aβ Induced Neuronal Cytotoxicity Under Oxidative Stress

Importantly, pretreatment of Sw-APP transfected SH-SY5Y cells with adiponectin at 10 µg/ml for 2 hours before exposure to H₂O₂ led to significant reduction of cytotoxicity under oxidative stress induced by H₂O₂ at concentration of 200 µM to 800 µM (Figure 5). Adiponectin of 10 µg/ml is at physiological concentration as documented previously [79]. The most marked protective effect was noted at 400 µM H₂O₂ as adiponectin pretreatment lowered cytotoxicity from 12.4 folds to 2.7 folds (p<0.0001). This strongly supports that adiponectin at physiological concentration of 10 µg/ml protects SH-SY5Y cells with Aβ neurotoxicity against cytotoxicity under oxidative stress. To ensure that adiponectin protection against cytotoxicity under oxidative stress induced by H₂O₂ in Sw-APP transfected SH-SY5Y cells was due to the effect of adiponectin binding to its cell surface receptors AdipoR1 and AdipoR2, we performed experiments using Sw-APP transfected SH-SY5Y cells with knockdown of AdipoR1 and AdipoR2 expression by RNA interference. The results showed that the protective effect of adiponectin against cytotoxicity under oxidative stress was lost in Sw-APP transfected SH-SY5Y cells with knockdown of AdipoR1 and AdipoR2, but preserved in control Sw-APP transfected SH-SY5Y cells without knockdown of either receptor (scramble RNA) (Figure 5B). This confirms that protective effect of adiponectin against Aβ induced neuronal cytotoxicity under oxidative stress requires binding of adiponectin to its cell surface receptors. With this, the possibility that adiponectin may directly interact with Aβ oligomers leading to blockade of Aβ oligomers-associated cytotoxic effects is unlikely.

Adiponectin Protection against Aβ Induced Neuronal Cytotoxicity Under Oxidative Stress Involved APPL1-dependent AMPK Activation

We further investigated the mechanisms of neuroprotection by adiponectin against Aβ neurotoxicity. As important cellular functions of adiponectin are exerted via APPL1-mediated AMPK activation leading to increased intracellular pAMPK, we proceeded to study whether protective effect of adiponectin against Aβ neurotoxicity involved APPL1-dependent AMPK activation. Using Sw-APP transfected SH-SY5Y cells with knockdown of APPL1 expression by RNA interference (Figures 6A and 6B), we found that the neuroprotective effect of adiponectin against cytotoxicity under H₂O₂ induced oxidative stress was lost in cells with knockdown of APPL1 expression but preserved in cells without knockdown of APPL1 expression (Figure 6C). These suggest that

![Figure 4. Cytotoxicity of SH-SY5Y cells transfected with empty vector, wild-type APP and the Swedish-APP mutant. A. LDH assay revealed that the basal cytotoxicity of Sw-APP transfected (Sw-APP), wt-APP transfected (APP) and empty-vector transfected (vector) SH-SY5Y cells are indifferent. B. Sw-APP transfected (Sw-APP) cells had increased cytotoxicity under oxidative stress induced by hydrogen peroxide (500 µM) than wt-APP transfected (APP) (p<0.0001) which had increased cytotoxicity under oxidative stress than empty-vector transfected (vector) cells (p<0.0001). Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test. doi:10.1371/journal.pone.0052354.g004](Image 58x297 to 297x730)
Figure 5. Adiponectin protection against cytotoxicity of SH-SY5Y cells transfected with Swedish-APP (Sw-APP) mutant under oxidative stress. (A) Percentage cytotoxicity of SH-SY5Y cells transfected with Sw-APP mutant under oxidase stress by different concentrations of hydrogen peroxide (H2O2) without and with pretreatment of adiponectin at 10 µg/ml expressed as fold of cytotoxicity relative to cytotoxicity without exposure to H2O2 (untreated, fold = 1) were shown on y-axis. In all three H2O2 concentrations, percentage cytotoxicity were significantly reduced with adiponectin pretreatment (p<0.05 for 200 µM H2O2, p<0.0001 for 400 µM H2O2 and p<0.05 for 800 µM H2O2). (--) ADN = without adiponectin pretreatment, (+) ADN = with adiponectin pretreatment. Results shown were mean values from 5 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test. (B) Using Sw-APP transfected SH-SY5Y cells with knockdown of AdipoR1 or AdipoR2 expression for the cytotoxicity experiments, it was observed that the protective effect of adiponectin against cytotoxicity under oxidative stress was preserved in control Sw-APP transfected SH-SY5Y cells without knockdown of AdipoR1 or AdipoR2 (scramble RNA+ADN+400 µM H2O2), but lost in cells with knockdown of AdipoR1 (AdipoR1 siRNA+ADN+400 µM H2O2) or AdipoR2 (AdipoR2 siRNA+ADN+400 µM H2O2) expression. This confirms that protective effect of adiponectin against cytotoxicity under oxidative stress in Sw-APP transfected SH-SY5Y cells required binding of adiponectin to its cell surface receptors AdipoR1 or AdipoR2. untreated = Sw-APP transfected SH-SY5Y cells without exposure to H2O2, 400 µM H2O2 = Sw-APP transfected SH-SY5Y cells exposed to 400 µM H2O2, ADN+400 µM H2O2 = Sw-APP transfected SH-SY5Y cells pretreated with adiponectin before exposure to 400 µM H2O2. Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test.

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neuroprotective effect of adiponectin against Aβ mediated neuronal cytotoxicity under oxidative stress is dependent on APPL1. In addition, Western blot analysis of cell lysates revealed that Sw-APP transfected SH-SY5Y cells pretreated with adiponectin before exposure to H2O2 had higher pAMPK level than Sw-APP transfected SH-SY5Y cells without adiponectin pre-

Figure 6. Adiponectin neuroprotection against cytotoxicity under oxidative stress is mediated via APPL1-dependent AMPK activation. A. Western blot analysis of control Swedish-APP mutant transfected SH-SY5Y cells (Sw-APP) and similar cells transfected with scramble RNA (Sw-APP+scramble RNA) revealed APPL1 expression. Successful knockdown of APPL1 expression by RNA interference was shown on immunoblotting of cell lysate of Sw-APP transfected SH-SY5Y cells transfected with short interference RNA for APPL1 (Sw-APP+siRNA). B. Image-J analysis confirmed successful knockdown of APPL1 expression in Sw-APP transfected SH-SY5Y cells by siRNA compared to control cells transfected with scramble RNA (p<0.0001). Transfection experiments were performed 3 times with consistent results. C. Neuroprotective effect of adiponectin against cytotoxicity under oxidative stress (400 μM H2O2) was lost in Sw-APP transfected SH-SY5Y cells with knockdown of APPL1 expression (siRNA+ADN+400 μM H2O2), but preserved in cells without knockdown of APPL1 expression (scramble siRNA+ADN+400 μM H2O2, p<0.0001). This suggests that adiponectin neuroprotection against Aβ-induced cytotoxicity under oxidative stress requires APPL1. Fold of percentage cytotoxicity relative to cells not exposed to H2O2 (untreated, fold = 1) was shown on y-axis. untreated = Sw-APP transfected SH-SY5Y cells without exposure to H2O2, 400 μM H2O2 = Sw-APP transfected SH-SY5Y cells exposed to 400 μM H2O2, ADN+400 μM H2O2 = Sw-APP transfected SH-SY5Y cells pretreated with adiponectin before exposure to 400 μM H2O2. Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test. D. Adiponectin induced AMPK activation. Western blot analysis of cell lysates from Sw-APP transfected SH-SY5Y cells exposed to oxidative stress (400 μM H2O2) demonstrated that cells pretreated with adiponectin (ADN) had increased phosphorylated AMPK (pAMPK) compared to cells without adiponectin pretreatment whereas APPL1 and total AMPK levels were similar. This strongly suggests that adiponectin neuroprotection against cytotoxicity under oxidase stress in Sw-APP transfected SH-SY5Y cells is mediated via APPL1-dependent AMPK activation. Western blot analysis were performed for 3 independent cultures with consistent results.

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treatment, while total AMPK level was similar (Figure 6D). These together suggest that the protective effect of adiponectin against Aβ induced neuronal cytotoxicity under oxidative stress is mediated via APPL1-dependent AMPK activation.

Human Neuroblastoma Cells Transfected with Sw-APP Mutant and wt-APP had Increased NF-κB Activation

Western blot analysis of nuclear extract for p65 component of NF-κB (NF-κB p65) revealed that Sw-APP transfected SH-SY5Y cells had higher NF-κB p65 level than wt-APP transfected cells (p<0.0001), which had higher NF-κB p65 level than empty-vector transfected cells (p<0.001) (Figure 7). These suggest that Aβ overproduction and Aβ neurotoxicity lead to increased NF-κB activation, consistent with the concept that neuroinflammation is one of the pathogenetic mechanisms in AD.

Figure 7. Western blot for nuclear factor-kappa B (NF-κB) activation. A. SH-SY5Y cells transfected with the Swedish-APP mutant (Sw-APP) had significantly higher level of nuclear NF-κB p65 than cells transfected with wild-type APP (APP), which had higher level of nuclear NF-κB p65 than cells transfected with empty-vector (vector). The control nuclear protein was histone 3. B Image J and statistical analysis confirmed statistically significant difference in the level of NF-κB activation. Sw-APP transfected SH-SY5Y cells under greatest Aβ neurotoxicity had the highest level of nuclear NF-κB p65 more than that of wt-APP transfected cells (p<0.0001), which had higher level of NF-κB p65 than empty-vector transfected cells without Aβ neurotoxicity (p<0.001). Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test.

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Figure 8. Adiponectin (ADN) suppressed NF-κB activation. A. Western blot of nuclear extracts revealed that SH-SY5Y cells transfected with Swedish-APP mutant had high level of NF-κB p65 signifying NF-κB activation (lane 1), and treatment with adiponectin (ADN) at 10 µg/ml for 2 hours resulted in reduced NF-κB activation (lane 2). Sw-APP transfected cells exposed to 400 µM H₂O₂ for 2 hours had high level of NF-κB activation (lane 3), and importantly Sw-APP transfected cells pretreated with adiponectin at 10 µg/ml for 2 hours before exposure to 400 µM H₂O₂ had reduced NF-κB activation (lane 4). Control nuclear protein was histone H3. B. Statistical analysis of imaging J values revealed that Sw-APP transfected SH-SY5Y cells (Sw-APP) had significant reduction level of NF-κB activation after treatment with ADN (Sw-APP+ADN, p<0.0005). Consistently, Sw-APP transfected SH-SY5Y cells exposed to oxidative stress of 400 µM H₂O₂ (Sw-APP+400 µM H₂O₂) had significantly reduced level of NF-κB activation with adiponectin pretreatment at 10 µg/ml for 2 hours before exposure to oxidative stress of 400 µM H₂O₂ (Sw-APP+AND+400 µM H₂O₂, p<0.0005). This raises the possibility that one mechanism underlying adiponectin neuroprotection against Aβ neurotoxicity-induced cytotoxicity under oxidative stress is suppression of NF-κB activation. Results shown were mean values from 3 independent cultures, and compared by one-way ANOVA using Newman-Keuls multiple comparison test.

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NF-κB Activation was Reduced in Sw-APP Transfected Human Neuroblastoma Cells Pretreated with Adiponectin

We further studied whether adiponectin might suppress NF-κB activation especially adiponectin is known to possess anti-inflammatory action. Western blot analysis of nuclear fraction of Sw-APP transfected SH-SY5Y cells revealed that cells treated with adiponectin at 10 μg/ml for 2 hours had significantly reduced NF-κB activation compared to untreated cells (p<0.0001) (Figure 8A and 8B). Similar reduction of NF-κB activation in Sw-APP transfected SH-SY5Y cells exposed to H₂O₂ induced oxidative stress cells was observed with adiponectin pretreatment before exposure to H₂O₂ (p<0.0001) (Figure 8A and 8B). These raise the possibility that one of the mechanisms underlying adiponectin protection against Aβ neurotoxicity was suppression of NF-κB activation, hence attenuating neuroinflammation.

Discussion

Aβ inhibits enzymes of mitochondrial oxidative phosphorylation and key enzymes of Krebs cycle, leading to mitochondrial dysfunction, reduced ATP production (energy failure), and generation of ROS and reactive nitrogen species (RNS) such as superoxides and nitrates. ROS and RNS contribute to oxidative stress which causes oxidation and nitration of proteins, nucleic acids and lipids leading to mitochondrial dysfunction, altered Ca²⁺ homeostasis, membrane damage and altered proteasome function with abnormal protein accumulation [49]. ROS also activate genes involved in inflammatory pathways such as IL-6, interferon-gamma (IFN-γ) and inducible nitric oxide synthase (iNOS) [17]. Activation of iNOS leads to excess endogenous nitric oxide (NO) which causes S-nitrosylation of dynamin-related protein-1, resulting in mitochondrial fission, synaptic loss and neuronal damage [50]. Hence, oxidative stress causes cellular dysfunctions, neuronal degeneration and death [49,51]. Importantly, increased oxidative stress occurs early in AD [51–53].

We demonstrated that Sw-APP transfected human neuroblastoma cells over-producing Aβ had increased susceptibility to cytotoxicity under oxidative stress. This supports that Aβ neurotoxicity mediated oxidative stress and mitochondrial dysfunction play important roles in AD pathogenesis [26,49,51]. The Sw-APP transfected SH-SY5Y cells had higher secreted Aβ oligomers level in culture medium and intracellular Aβ oligomers level compared to empty-vector transfected cells. We are uncertain whether the increased susceptibility to cytotoxicity under oxidative stress is due to the extracellular or intracellular Aβ oligomers, but probably the latter. Our observation that AMPK activation was increased in Sw-APP transfected cells compared to wt-APP and empty-vector transfected cells may imply that increased pAMPK level from AMPK activation is beneficial for neuronal survival under Aβ neurotoxicity. AMPK is a sensor of cellular energy level and is activated by increase in AMP to ATP ratio, hence functions to preserve cellular energy. AMPK activation upregulates ATP-producing catabolic pathways such as uptake and metabolism of glucose and fatty acids, and downregulates ATP-consuming anabolic pathways such as synthesis of glycogen, cholesterol and proteins [54]. It is likely that Aβ neurotoxicity in the Sw-APP transfected neuroblastoma cells induces mitochondrial dysfunction leading to decreased ATP level, hence stimulates AMPK activation. Treatment of cultured embryonic rat hippocampal neurons under glucose deprivation with a potent AMPK activator led to improved cell survival. Similar beneficial effect was also found in neurons exposed to Aβ peptide, sodium cyanide (mitochondrial toxin) and glutamate [55]. However, Thornton et al. reported that treatment of primary mouse cortical neurons with Aβ42 induces AMPK activation which leads to increased tau phosphorylation. This pathway of Aβ42 induced AMPK activation involves the N-methyl-D-aspartate (NMDA) receptor as AMPK activation is inhibited by memantine, a partial NMDA receptor antagonist used in treatment of AD [36]. Consistently, Vingirides et al. reported that activated AMPK (pAMPK) is abnormally accumulated in cerebral neurons of patients with tauopathies including AD. In AD, pAMPK is accumulated in neuropil threads and dystrophic neurites surrounding amyloid plaques in >90% of neurons bearing neurofibrillary tangles and pre-tangles. The investigators proposed that AMPK might regulate neurodegeneration by controlling tau phosphorylation [57]. Whether AMPK activation leads to tau phosphorylation cannot be addressed in our study.

Une et al. reported that plasma adiponectin level was significantly higher in mild cognitive impairment (MCI) and AD patients than normal controls (NC), whereas CSF adiponectin level was significantly higher in MCI than NC [58]. This increased adiponectin level may be a compensatory response to protect against neurodegeneration in MCI or early AD. However, van Himbergen et al. studied 840 dementia-free Framingham Heart Study participants over a mean follow-up period of 13 years and reported that 159 persons developed dementia (125 AD); and after adjustment of other risk factors, only adiponectin in women was associated with an increased risk of all-cause dementia [hazard ratio (HR) 1.29, p = 0.054] and AD (HR 1.44, p = 0.050) per 1 standard deviation increase in adiponectin level. In AD, women with baseline adiponectin levels above the median had a higher risk of all-cause dementia (HR 1.63, p = 0.04) and AD (HR 1.87, p = 0.01) compared to those with adiponectin levels below median [59]. The exact role of adiponectin in human cognitive functions awaits further clarification. Importantly, our results demonstrated that adiponectin at a physiological concentration of 10 μg/ml (physiological serum concentration 3–30 μg/ml) is protective against cytotoxicity of SH-SY5Y cells exposing Sw-APP mutant under H₂O₂ induced oxidative stress; and this protective effect of adiponectin against Aβ neurotoxicity is dependent on APPL1, likely mediated via APPL1-dependent AMPK activation. APPL1 plays important role as an endosomal adaptor protein in various cellular signaling pathways [38,60]. AMPK activation promotes ATP producing catabolic pathways including glucose uptake and metabolism which is diminished in AD brain with insulin deficiency and resistance [21]. AMPK activation protects and confers survival benefits to neurons against oxidative, metabolic and excitotoxic stress [54–55]. Resveratrol, a neuroprotective plant polyphenol, promotes neurite outgrowth and mitochondrial biogenesis in neurons via AMPK activation [61]. In addition, adiponectin-mediated activation of AMPK, p38MAPK and Rab5 leads to increased glucose transporter 4 (Glut4) membrane translocation [62] which also promotes neuronal glucose uptake and metabolism. Insulin is important for neuronal function and survival, and reduced cerebral insulin and insulin-like growth factor (IGF-1) levels, as well as impaired neuronal insulin signaling are noted in AD brain [63–64]. Both the expression and function of insulin and IGF-1 deteriorate with progression of AD [57], and intranasal insulin improves cognition and modulates Aβ in early AD [65]. The insulin-sensitizing action of adiponectin [33,60] may be another mechanism of neuroprotection in AD which cannot be addressed in our study.

Neuroinflammation is increasingly recognized as an important element in AD [22,24,66–67]. Reactive astrocytes in close proximity to Aβ plaques and activated microglia secrete inflammatory mediators including IL-1β, TNF-α, IL-6, interleukin...
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18 IL-18) and iNOS which generate free radicals such as NO. In addition, Aβ undergoes non-enzymatic glycation to form advanced glycation end products (AGEs) which bind to receptor for AGEs (RAGE). This AGEs/RAGE signaling activity upregulates activity of the transcription factor, nuclear factor kappa-B (NF-kB) [60]. NF-kB activation is a central event of neuroinflammation in AD [71], and is predominantly found in neurons and glial cells at regions surrounding Aβ plaques in AD brain [72–75]. NF-kB activation triggers expression of pro-inflammatory molecules including cytokines and chemokines which contribute to neuronal cytotoxicity in AD [71]. This is consistent with our observation that Sw-APP transfected and wt-APP transfected neuroblastoma cells overproducing Aβ have increased NF-kB activation than empty-vector transfected cells not overproducing Aβ. Importantly, NF-kB activation in Sw-APP transfected neuroblastoma cells diminished with adiponectin treatment in parallel with its protective effect against cytotoxicity under oxidative stress. This raises the possibility that suppression of NF-kB activation is one mechanism underlying neuroprotective effect of adiponectin against Aβ-induced neuronal cytotoxicity under oxidative stress. Adiponectin was recently reported to block IL-18 mediated endothelial cell death [76]. Treatment of endothelial cells with IL-18 suppressed Akt (protein kinase B) phosphorylation and its associated kinase activity [77], and induced IkB kinase (IKK)-NF-kB-dependent phosphatase and tensin homolog (PTEN) activation, hence promoted endothelial cell death [76]. Pretreatment with adiponectin stimulated APPL1-dependent AMPK activation, reversed Akt inhibition, inhibited IKK-NF-kB dependent PTEN expression in an AMPK-dependent manner, hence blocked IL-18 mediated endothelial cell death [76]. This is consistent with the cytoprotective effect of adiponectin via AMPK activation which inhibits IKK-NF-kB dependent-PTEN pathway [60]. IL-18 is upregulated in AD brain [78] and IL-18 production by peripheral blood cells is increased in AD patients, the level of which correlates with cognitive impairment [79]. These suggest that IL-18 related inflammatory pathways are exacerbated in AD, which can be attenuated by adiponectin. In addition, adiponectin was reported to protect neurons against ischemic-reperfusion injury in a rat model of stroke through anti-inflammatory action via inhibition of NF-kB activation [80].

This is the first report that adiponectin is protective in human neuroblastoma cells with Aβ neurotoxicity. Adiponectin has been reported to be neuroprotective in 1) SH-SY5Y cells against MPP+-induced cytotoxicity [81], 2) SH-SY5Y cells against acetadyldehydro-induced apoptosis [48], 3) a mouse model of epilepsy against kainic acid-induced excitotoxicity [82] and 4) a rat model of stroke against cerebral ischemia-reperfusion injury [80]. It is encouraging that a recent study reported that intense expression of AdipoR1 was noted in the hypothalamus and the nucleus basalis of Meynert in basal forebrain which is frequently affected in AD [83]. Further studies are needed to confirm the neuroprotective effect of adiponectin in Aβ neurotoxicity, which bears potential for novel therapies against AD. Interestingly, another adipokine, leptin has been shown to reduce Aβ levels in neuronal cells via AMPK activation [84], and reduce pathology and improve memory in a transgenic mouse AD model [85].

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Author Contributions

Conceived and designed the experiments: KHC JSCK PWLH KSLK SKC. Performed the experiments: OYC JSCK JWMH VYG. Analyzed the data: KHC JSCK OYC KKYC SKC. Contributed reagents/materials/analysis tools: KHC JSCK PWLH KSLK. Wrote the paper: KHC KSLL.

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