S14G-humanin restored cellular homeostasis disturbed by amyloid-beta protein

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Research Highlights

(1) This study sought to investigate the inhibitory effects of S14G-humanin on amyloid-beta protein-induced hippocampal neuronal injury from the perspectives of cell membrane fluidity, calcium homeostasis, generation of reactive oxygen species, and mitochondrial function.
(2) This study concluded that S14G-humanin blocks the action of amyloid-beta protein on neuronal membrane and restores perturbed cellular homeostasis, thereby alleviating the neurotoxic effects of amyloid-beta protein on hippocampal neurons.

Abstract

Humanin is a potential therapeutic agent for Alzheimer's disease, and its derivative, S14G-humanin, is 1 000-fold stronger in its neuroprotective effect against Alzheimer's disease-relevant insults. Although effective, the detailed molecular mechanism through which S14G-humanin exerts its effects remains unclear. Data from this study showed that fibrillar amyloid-beta 40 disturbed cellular homeostasis through the cell membrane, increasing intracellular calcium, generating reactive oxygen species, and decreasing the mitochondrial membrane potential. S14G-humanin restored these responses. The results suggested that S14G-humanin blocked the effects of amyloid-beta 40 on the neuronal cell membrane, and restored the disturbed cellular homeostasis, thereby exerting a neuroprotective effect on hippocampal neurons.

Key Words

neural regeneration; Alzheimer's disease; amyloid-beta protein; wild type humanin; S14G-humanin; reactive oxygen species; mitochondrial membrane potential; grants-supported paper; neurodegeneration; neuroregeneration

INTRODUCTION

One of the distinct characteristics of Alzheimer's disease is the extensive presence of amyloid plaques in the brain. These plaques consist mainly of amyloid-beta (Aβ) protein derivatives. Aβ, the key factor of cellular changes in Alzheimer's disease brains, leads to the deregulation of calcium homeostasis, generation of free radicals, and dysfunction of mitochondria[1-2]. Disruption of intracellular Ca^{2+} homeostasis has been reported to be one of the mechanisms of Aβ-mediated neurotoxicity in Alzheimer's disease. This theory is supported by experimental studies that reveal significant alterations in levels of proteins and genes directly involved in neuronal Ca^{2+} signaling in sporadic and familial Alzheimer's disease[3-4]. Moreover, Aβ can promote cellular Ca^{2+} overload by inducing membrane-asso-

ciated oxidative stress and by inserting into the plasma membrane and forming ion conducting pores[5].

Accumulating evidence suggests that oxidative stress is an early event in Alzheimer's disease. The early involvement of oxidative stress in this disease is demonstrated by decreased levels of antioxidant enzymes and increased oxidative modifications of lipids, proteins, and nucleic acids in brains of Alzheimer's disease patients, and also in cellular and animal models of Alzheimer's disease[6-10]. In Alzheimer's disease patients and postmortem Alzheimer's disease brain tissues, activities of three key enzymes of the respiratory chain complexes I, III, and IV are impaired resulting in the overproduction of reactive oxygen species[11]. Moreover, mitochondrial DNA isolated from the brains of Alzheimer's disease patients shows oxidative modifications containing 8-hydroxy-2-deoxyguanosine[12]. Further evidence has also suggested that Aβ disrupts the electron-transport chain, increases reactive oxygen species generation, causes mitochondrial damage, and prevents neurons from functioning normally[13].

The newly discovered neuroprotective peptide, humanin, may have potential as a therapeutic agent for Alzheimer's disease[14]. In vitro studies have revealed that humanin may effectively protect neuronal cells against the majority of all Alzheimer's disease-related pathological mechanisms, such as various genes associated with familial Alzheimer's disease, amyloid precursor protein, and neurotoxic Aβ peptides ($A\beta_{1-42}$, $A\beta_{1-43}$, and $A\beta_{25-35}$)[15-17]. Surprisingly, S14G-humanin, a derivative of humanin (via a substitution of Gly for Ser14 of humanin), enhances its neuroprotective activity against Alzheimer's disease-relevant insults by approximately 1 000-fold stronger, and is fully active at low nanomolar concentrations[18]. Intraperitoneal injection of S14G-humanin reverses the spatial memory and orientation impairment induced by the anticholinergic drug, 3-quinuclidinyl benzilate[19]. Furthermore, intracerebroventricular injection of S14G-humanin was shown to prevent Aβ-induced impairment of short-term and long-term memory in mice[20].

Although humanins can prevent Aβ-induced cell death, the exact mechanism by which they exert this effect remains unknown. Therefore, in the present study, we focused on how humanins may affect these cellular changes induced by Aβ. Hippocampal neuronal primary cultures exposed to Aβ were used as a cellular injury model. The effects of S14G-humanin on Aβ-induced neurotoxicity, and possible neuroprotective mechanisms, were then explored by measuring membrane fluidity,

calcium disturbance, reactive oxygen species elevation, and mitochondrial dysfunction.

RESULTS

S14G-humanin prevented the changes of membrane fluidity decreased by $A\beta_{40}$

Because both humanin and $A\beta_{40}$ affect cellular activity via the cell membrane, membrane fluidity was thus one of the most important parameters to measure. Membrane fluidity of hippocampal cells was assessed using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), after treatment with different working peptides. Greater membrane polarization (P) values indicated lower membrane fluidity. Fibrillar $A\beta_{40}$ decreased membrane fluidity in a concentration-dependent manner; and soluble $A\beta_{40}$ did not change this phenomenon (Figure 1). Both humanin and $A\beta_{40}$ decreased membrane fluidity, but not as much as fibrillar $A\beta_{40}$ (Figure 1). Although membrane fluidity was reduced by $A\beta_{40}$ + humanin treatment, it was not as potent as fibrillar $A\beta_{40}$ itself (Figure 1). $A\beta_{40}$ + S14G-humanin did not disturb membrane fluidity, suggesting that S14G-humanin maintained $A\beta_{40}$-mediated dysfunction of membrane fluidity. Bovine serum albumin (negative control) did not change membrane fluidity.
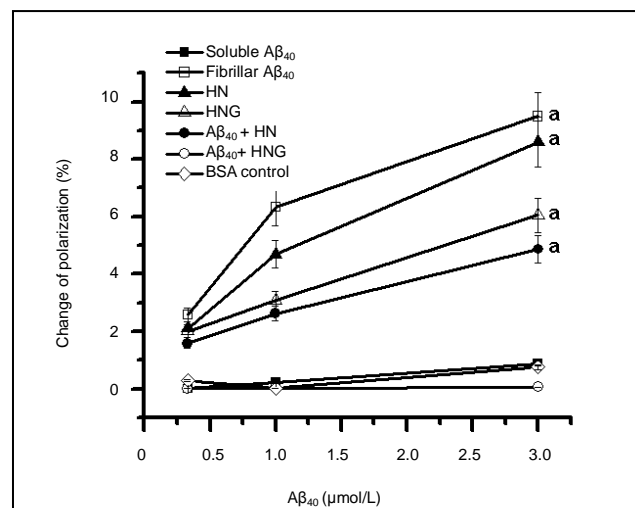
Figure 1　Alterations in membrane fluidity of hippocampal cells by peptides.

Membrane fluidity was measured (via the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene), after a 12-day exposure to fibrillar $A\beta_{40}$, soluble $A\beta_{40}$, humanin (HN), S14G-humanin (HNG), $A\beta_{40}$ + HN, $A\beta_{40}$ + HNG, or bovine serum albumin (BSA). Three independent experiments were performed in duplicate, and the data are expressed as mean ± SD (One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls' multiple range test. [a]$P < 0.01$, vs. BSA).

S14G-humanin restored Aβ$_{40}$-induced disruption of [Ca^{2+}]i homeostasis

Interaction of Aβ$_{40}$ with the plasma membrane results in elevated intracellular calcium (Ca^{2+}) concentrations ([Ca^{2+}]i)[21]. Furthermore, Ca^{2+} plays an important role in cell activities. Therefore, we investigated the effect of the different peptides on [Ca^{2+}]i (using the Fluo-3/AM dye) in hippocampal neuronal cultures. KCl (positive control) increased [Ca^{2+}]i (by 2.5-fold) more than the tested peptides (Figure 2). Soluble Aβ$_{40}$ and both humanins (humanin and S14G-humanin) did not significantly change [Ca^{2+}]i (Figure 2). Fibrillar Aβ$_{40}$ increased [Ca^{2+}]i 1.5-fold. Aβ40 + humanin increased calcium levels as much as fibrillar Aβ$_{40}$, however Aβ$_{40}$ + S14G-humanin did not have an effect (Figure 2). These results showed that S14G-humanin blocked the ability of fibrillar Aβ$_{40}$ to elevate [Ca^{2+}]i, thus suggesting that S14G-humanin restored Aβ$_{40}$-mediated dysfunction of calcium homeostasis.
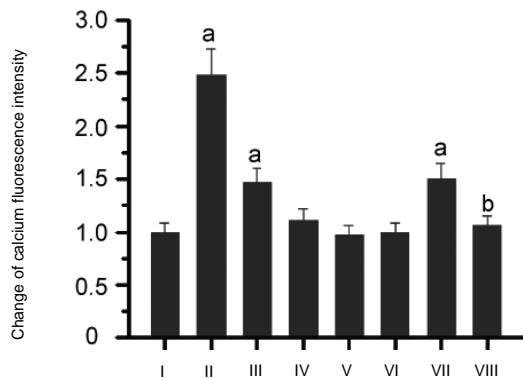

Figure 2   Changes of intracellular calcium concentrations ([Ca^{2+}]i) in hippocampal neurons after peptide treatment.

I: Hank's buffer; II: KCl; III: fibrillar Aβ$_{40}$; IV: soluble Aβ$_{40}$; V: humanin; VI: S14G-humanin; VII: Aβ$_{40}$ + humanin; VIII: Aβ$_{40}$ + S14G-humanin. The fluorescence ratio of calcium was measured 30 seconds after treatment with different peptides. Three independent experiments were performed in duplicate, and the data are expressed as mean ± SD. One-way analysis of variance followed by Student-Newman-Keuls' multiple range test was performed. [a]$P < 0.01$, vs. Hank's buffer; [b]$P < 0.01$, vs. fibrillar Aβ$_{40}$.

Humanins prevented Aβ$_{40}$-mediated increases of intracellular reactive oxygen species levels

Intracellular reactive oxygen species levels were increased, approximately 2-fold more with glutamate treatment compared with Hank's buffer (Figure 3). Reactive oxygen species levels tripled when cells were exposed to fibrillar Aβ$_{40}$ (Figure 3). Soluble Aβ$_{40}$ also significantly increased the generation of reactive oxygen species generation (Figure 3). However, neither humanin combined with Aβ$_{40}$ significantly increased reactive oxy-

gen species (Figure 3). These results suggested that humanins prevented the fibrillar Aβ$_{40}$-mediated production of reactive oxygen species.
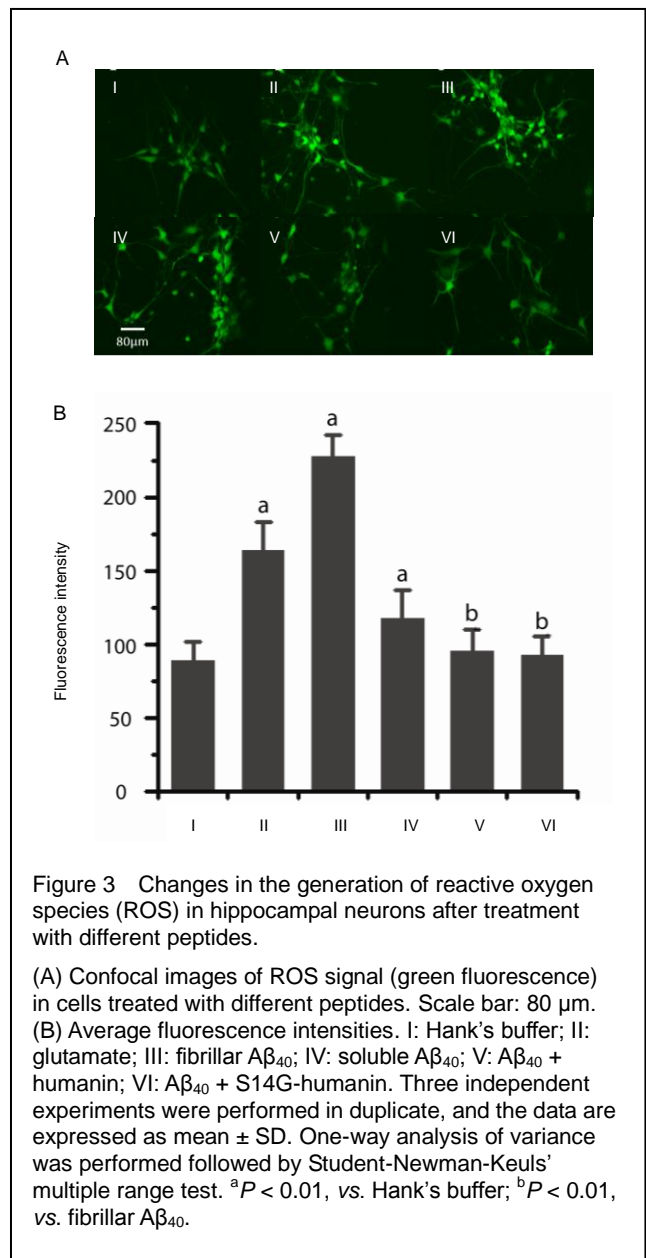

Figure 3   Changes in the generation of reactive oxygen species (ROS) in hippocampal neurons after treatment with different peptides.

(A) Confocal images of ROS signal (green fluorescence) in cells treated with different peptides. Scale bar: 80 μm. (B) Average fluorescence intensities. I: Hank's buffer; II: glutamate; III: fibrillar Aβ$_{40}$; IV: soluble Aβ$_{40}$; V: Aβ$_{40}$ + humanin; VI: Aβ$_{40}$ + S14G-humanin. Three independent experiments were performed in duplicate, and the data are expressed as mean ± SD. One-way analysis of variance was performed followed by Student-Newman-Keuls' multiple range test. [a]$P < 0.01$, vs. Hank's buffer; [b]$P < 0.01$, vs. fibrillar Aβ$_{40}$.

Mitochondrial membrane potential (Δωm) was reduced in all peptide groups

We used fluorescence probe JC-1 to monitor Δωm in hippocampal neuronal cells. The control group exhibited red fluorescence (J-aggregate), indicating mitochondria with normal function (Figure 4A). Glutamate and fibrillar Aβ$_{40}$ induced green fluorescence (J-monomer) (Figure 4A), suggesting mitochondria with decreased Δωm. The soluble Aβ$_{40}$, Aβ$_{40}$ + humanin, and Aβ$_{40}$ + S14G-humanin groups (Figure 4A) had stronger green fluorescence than red fluorescence, suggesting mitochondria with decreased Δωm. Quantitative analysis revealed that all peptide groups significantly ($P < 0.01$) decreased Δωm

(Figure 4B). In pite of the 12-day incubation of $A\beta_{40}$ + humanin/S14G-humanin, $\Delta\omega m$ dissipation still occurred (Figure 4B). However, $\Delta\omega m$ was not reduced as much by soluble $A\beta_{40}$ +humanin/S14G-humanin as compared with fibrillar $A\beta_{40}$ (Figure 4B).

Figure 4   Decrease in mitochondrial membrane potential ($\Delta\omega m$) in hippocampal neurons treated with different peptides.

(A) Confocal images of JC-1 signal showing changes in $\Delta\omega m$ in different groups. Red fluorescence indicates J aggregates, green fluorescence-J-monomers, and orange fluorescence indicates the merge of both colors. (B) The average fluorescence ratio at 590 nm/530 nm in different peptide groups. I: Hank's buffer; II: glutamate; III: fibrillar $A\beta_{40}$; IV: soluble $A\beta_{40}$; V: $A\beta_{40}$ + humanin; VI: $A\beta_{40}$ + S14G-humanin. Three independent experiments were performed in duplicate, and the data are expressed as mean ± SD. One-way analysis of variance was performed followed by Student-Newman-Keuls' multiple range test. [a]$P < 0.01$, *vs.* Hank's buffer; [b]$P < 0.01$, *vs.* fibrillar $A\beta_{40}$.

DISCUSSION

Our data indicate that fibrillar $A\beta_{40}$ decreases membrane fluidity in a concentration dependent manner. However, membrane fluidity is not perturbed after a 12-day incubation of $A\beta_{40}$ + S14G-humanin, suggesting that S14G-humanin maintains $A\beta_{40}$-mediated dysfunction of membrane fluidity. Maintenance of membrane fluidity is linked with Ca^{2+} and thus, may play an important roles in

cells. Neurons use Ca^{2+} signals to control membrane excitability, trigger release of neurotransmitters, mediate activity-dependent changes in gene expression, and modulate neuronal growth, differentiation and the transition to apoptosis. $A\beta_{40}$ was reported to increase cytosolic Ca^{2+} concentrations when added to media of cultured cells[22]. Whether humanins in the presence of $A\beta_{40}$ inhibit $[Ca^{2+}]i$ remains unknown.

Excessive and sustained elevations in Ca^{2+} induce free-radical production by altering mitochondrial oxidative phosphorylation and activating oxygenases, resulting in perturbed Ca^{2+} homeostasis[23]. This phenomena may contribute to increased oxidative stress in neurons in Alzheimer's disease, resulting in a self-amplifying cascade of free-radical- and Ca^{2+}-mediated degenerative processes. Overwhelming evidence suggests that $A\beta$ increases the production of reactive oxygen species in cells[6-11]. Balancing this production with its consumption may be one of the mechanisms by which humanin protects neurons. Therefore, we measured changes in levels of reactive oxygen species in hippocampal cells treated by different peptides.

Mitochondria serve as high-capacity Ca^{2+} sinks, which allows them to stay in tune with changes in cytosolic Ca^{2+} loads, and facilitate in maintaining cellular Ca^{2+} homeostasis that is required for normal neuronal function[24-25]. Furthermore, because mitochondria are a major intracellular source of reactive oxygen species, they are particularly vulnerable to oxidative stress. Extensive literature supports a causative role of mitochondrial dysfunction and oxidative stress in the pathogenesis of Alzheimer's disease[2, 4, 6-7]. In the present study, because reactive oxygen species generation was inversely correlated with $\Delta\omega m$ we investigated the changes in $\Delta\omega m$ that indicates mitochondrial function.

Our data suggest that S14G-humanin functions through the cellular membrane to suppress the elevated levels of calcium and reactive oxygen species induced by fibrillar $A\beta_{40}$. Although S14G-humanin restored Ca^{2+} and reactive oxygen species homeostasis, it did not facilitate the dissipated potential mitochondria[26]. A recent hypothesis predicts that in the early stage of Alzheimer's disease, $A\beta$ enters mitochondria and induces the generation of reactive oxygen species and subsequently, oxidative stress[27]. Accordingly, mitochondria may be the main target of $A\beta$. Moreover, changes of mitochondrial function may be more sensitive than reactive oxygen species generation and Ca^{2+} elevation. Therefore, this hypothesis may explain why S14G-humanin restored

$A\beta_{40}$-mediated perturbation of the homeostatic production of reactive oxygen species and Ca^{2+}, but not mitochondrial function.

Reactive oxygen species generated by mitochondria have several cellular targets, including mitochondrial components themselves (*i.e.*, lipids, proteins, and DNA)[24, 27]. Mitochondria also serve as high-capacity Ca^{2+} sinks to aid for maintaining cellular Ca^{2+} homeostasis, which is required for normal neuronal function[25]. Conversely, excessive Ca^{2+} uptake into mitochondria debilitates mitochondrial function by increasing reactive oxygen species production, inhibiting ATP synthesis, and inducing the mitochondrial permeability transition pore[26]. $A\beta$ has been previously shown to depend on functional mitochondria to induce toxicity[28]. Previous *in vitro* studies also showed that $A\beta$ peptides decreased the Δωm in the presence of Ca^{2+}[29]. Altogether, these results suggest a clear association between $A\beta$, mitochondrial dysfunction, reactive oxygen species and alteration of Ca^{2+} homeostasis: Neuronal survival may depend on a delicate balance between mitochondrial function, calcium homeostasis, reactive oxygen species production, amyloid metabolism and cell signaling[30].

Overall, fibrillar $A\beta_{40}$ functions through the cellular membrane on mitochondria to increase intracellular levels of Ca^{2+} and reactive oxygen species, and S14G-humanin blocks this effect on the membrane, partially protecting mitochondria thus restoring perturbed cellular homeostasis. Previous studies have indicated that humanin exerts neuroprotective effects through long-term potentiation regulation related mechanisms[31-32]. Intranasal administration of S14G-humanin to triple transgenic male mice improved cognitive impairment[14]. $A\beta$ degradation is also increased with this drug, evidenced by elevated expression of neprilysin[14]. Intraperitoneal injection of S14G-humanin attenuated cognitive deficits, and reduced $A\beta$ loads and neuroinflammation. S14G-humanin may thus be used as a therapeutic agent for cognitive deficits and neuropathology associated with Alzheimer's disease[33]. Therefore, because S14G-humanin suppresses Alzheimer's disease-related insults under *in vivo* conditions, clinical application of humanins may be a promising candidate for Alzheimer's disease therapy[33-34].

MATERIALS AND METHODS

Design
An *in vitro* controlled cell culture experiment.

Time and setting
This study was performed at Henan Provincial People's Hospital of Zhengzhou University, China from October 2009 to February 2011.

Materials
Thirty female newborn Sprague-Dawley rats (< 1 day old and 8 g) were obtained from the Laboratory Animal Center of Henan Province, China (license No. SYXK (Yu) 2010-0001). All experimental protocols were approved by the Ethics Committee of Henan Provincial People's Hospital, China.

Methods
Peptide synthesis and purification
Humanin peptides, wild-type Humanin and S14G-Humanin, derived from human peptide sequence (NCBI accession No. AY029066), were synthesized with Fmoc chemistry using the solid phase peptide synthesis strategy[35]. Further purification occurred *via* high performance liquid chromatography[36], and was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry[36]. Purified wild-type Humanin and S14G-Humanin were freeze-dried, stored at –80°C, dissolved in 10 mg/mL bovine serum albumin (BSA; 27 mmol/L KCl, 137 mmol/L NaCl, 0.02% NaN_3, pH 7.2), and filtered (through 0.22 μm membranes).

Preparation of working peptides
Soluble $A\beta_{40}$ (AnaSpec, Fremont, CA, USA) was pre-treated for 1 hour with 100% trifluoroacetic acid at room temperature to obtain a chemically homogeneous sample. $A\beta_{40}$ was then dissolved in dimethyl sulfoxide (Calbiochem, Billerica, MA, USA) (10 mg/mL), stored at –20°C, and diluted in PBS immediately before use. Fibrillar $A\beta_{40}$ was derived from soluble $A\beta_{40}$ by a 12-day incubation at 37°C. Six different groups for working peptides were developed: soluble $A\beta_{40}$, fibrillar $A\beta_{40}$, humanin, S14G-humanin, $A\beta_{40}$ + humanin (incubated together for 12 days at 37°C) and $A\beta_{40}$ + S14G-humanin (incubated together for 12 days at 37°C). The molar ratio of humanins/$A\beta_{40}$ remained at 2.

Hippocampal neuron primary cultures
Hippocampal neurons were cultured, as previously described[29]. Briefly, postnatal Sprague-Dawley rats were sacrificed[34] and the hippocampi were dissected and incubated for 10 minutes in Hank's buffer (pH 7.4; containing 2.5 μg/mL trypsin). Cells were dissociated by gentle trituration using a fire-polished Pasteur pipette in cold Dulbecco's modified eagle's medium supplemented

with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). Cells were then filtered through a filter (53 mm diameter). A homogenous suspension of individual neurons was plated on petri-culture dishes, at a density of 1×10^5 mL, and maintained in neurobasal medium (Gibco) supplemented with L-glutamine, penicillin/streptomycin and 1% B-27 (Gibco) at 37°C with 5% CO_2. 6–8 day-old cultures (when 80% confluence was achieved) were utilized.

Measurement of membrane fluorescence anisotropy in hippocampal cells

Hippocampal cell pellets were resuspended and incubated with 0.5 µmol/L DPH for 20 minutes at 37°C, then washed and resuspended in Hank's buffer at 1×10^5 cells/mL. The labeled hippocampal cells were divided into two groups: (1) exposure to different working peptides (30 minutes at 37°C) followed by polarization ($P_{treated}$) measurement, and (2) exposure with PBS followed by $P_{untreated}$ (control) measurements. Bovine serum albumin was used as the control peptide. Humanin concentration used was 0.66, 2 and 6 µmol/L. The molar ratio of Humanins/$A\beta_{40}$ remained at 2[32].

Membrane fluidity of hippocampal cells was determined using the fluorescence probe DPH (Sigma, St. Louis, MO, USA)[37]. Due to its long rod-like structure and its non-polar properties, DPH aligns in parallel to the phospholipid fatty acyl chains of biological membranes, indicative of membrane fluidity character. Membrane fluidity is inversely correlated with membrane anisotropy. Steady-state fluorescence was measured by the 850 spectrofluorometer (Hitachi, Chiyoda, Tokyo, Japan) (excitation/emission of 362 nm/430 nm) at 37°C. P values were calculated from the following equation: $P = [I_{vv} – G$ IVH] / [I_{vv} + G IVH]. I_{vv} and IVH are the measured fluorescence intensities (with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively). G is termed as the grating correction factor, and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, equal to IHV/IHH. All experiments were done with multiple sets of samples and the data were shown as the changes of polarization (P%, [$P_{treated}$– $P_{untreated}$]/100$P_{untreated}$).

Measurement of [Ca²⁺]i

Calcium was monitored because of its important roles in neuron membrane excitability, release of neurotransmitters, gene expression, neuronal growth, differentiation, and apoptosis[25]. The membrane-permeable dye, Fluo-3/AM (Molecular Probes, Carlsbad, CA, USA), was used to measure the changes of [Ca²⁺]i[37]. Once taken

up by the cells, the ester was hydrolyzed by an esterase in the cytoplasm, releasing the free acid (hydrolyzed) form of the dye (Fluo-3), which is sensitive to calcium. The fluorescence of Fluo-3 increased with the binding of Ca^{2+}. Hippocampal cells (6–8 days old) were labeled with 4 mmol/L fluo-3/AM (25 minutes at 37°C in the dark). Real time fluorescence measurements of Ca^{2+} were performed in the Petri-dish using a laser confocal scanning microscope (Leica TCS-SP2, Wetzlar, Germany) (excitation/emission of: 485 nm/530 nm). Once a stable baseline was captured, different peptides were added to observe the dynamic changes of Ca^{2+} in cells (for 10 minutes at room temperature). The concentration used for $A\beta_{40}$ and Humanin was 8 µmol/L and 16 µmol/L, respectively. KCl (30 mmol/L) or Hank's buffer were used as a positive or negative control, respectively. One hundred cells were acquired and analyzed for each condition.

Measurement of reactive oxygen species

Reactive oxygen species in hippocampal cells was measured because excessive and sustained Ca^{2+} elevations induce free-radical production (by altering mitochondrial oxidative phosphorylation and activating oxygenases)[24, 26]. The nonpolar cell-permeable fluorescence probe, 2',7'-dichlorodihydroflurorescein diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA, USA)[31] was used to measure intracellular reactive oxygen species. To detect reactive oxygen species accumulation, 6–8 day-old cells were first treated with different peptides for 12 hour at 37°C. The concentration of $A\beta_{40}$ and Humanin used was 8 µmol/L and 16 µmol/L, respectively. Glutamate (5 mmol/L) or Hank's buffer was used as a positive or negative control, respectively. Then cells were loaded with 5 µmol/L 5-(and-6)-chloromethyl(CM)-H_2DCFDA (CM-H_2DCFDA) (in the dark for 15 minutes). Once CM-H_2DCFDA entered the cells, the diacetate group was removed by intracellular esterase so that the fluorescence probes were trapped inside the cells. The fluorescence intensity of intracellular dichlorofluorescin diacetate (DCFDA) was a linear indicator of the amount of reactive oxygen species. Reactive oxygen species was quantified by measuring the fluorescent product in the cytosol at an excitation/emission of 488 nm/530–560 nm.

Determination of mitochondrial membrane potential (Δωm)

Mitochondria serve as high-capacity Ca^{2+} sinks and also serve as a major intracellular source of reactive oxygen species, which make mitochondria particularly vulnerable to oxidative stress. Extensive literature supports a causa-

tive role of mitochondrial dysfunction and oxidative stress in the pathogenesis of Alzheimer's disease[20, 23-24, 26]. Therefore, we investigated the changes in Δωm (indicative of mitochondrial function). Δωm was measured using 5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide probe (JC-1) (Invitrogen)[32]. JC-1 accumulated in the mitochondria, shifting fluorescence emission from green (JC-1 monomer, 530 nm) to red (J-aggregates, 590 nm). Consequently, mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio. 6–8 day-old hippocampal cells were incubated with different working peptides respectively (for 12 hours at 37°C). A 12 hour treatment with glutamate (5 mmol/L) or Hank's buffer was used as a positive or negative control, respectively. To measure Δωm, control and treated cells were labeled with 2 μmol/L JC-1 (30 minutes at 37°C, in the dark). Cell images were recorded at 590 nm and 530 nm *via* confocal microscopy. The ratio of the reading at 590nm/530 nm was considered as the relative Δωm value.

Statistical analysis

All data were expressed as mean ± SD, and analyzed by one-way analysis of variance followed by Student-Newman-Keuls' multiple range test with multiple comparison tests, accordingly. Statistical analyses were processed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). Significance was reached at values of $P < 0.05$.

Research background: Humanin can effectively protect neuronal cells against nearly all Alzheimer's disease insults, such as neurotoxic Aβ peptides (Aβ$_{1-42}$ and Aβ$_{1-43}$). S14G-humanin, a derivative of humanin, enhances its neuroprotective ability against Alzheimer's disease insults.

Research frontiers: Extensive extracellular amyloid plaques mainly consisting of amyloid-beta protein derivatives are present in the brains of Alzheimer's disease patients. Aβ can induce cellular death. Although humanins can block Aβ-induced cell death, the detailed molecular mechanism by which humanins induce neuroprotective effects against Aβ-induced damaged cells remains to be further clarified.

Clinical significance: S14G-humanin blocks the action of Aβ on neuronal membranes and protects mitochondria to restore perturbed cellular homeostasis, thereby exhibiting a potential and effective treatment for Alzheimer's disease.

Academic terminology: S14G-humanin-A derivative of humanin (substitution of Gly for Ser14 of humanin) with a neuroprotective ability, 1 000-fold stronger than humanin. S14G-humanin can effectively inhibit neurotoxic effects mediated by Alzheimer's disease-related pathogenic factors.

Peer review: Mechanisms of the newly therapeutic agent, hu-

manin, were further studied in the Alzheimer's disease rat. This paper addresses that Aβ works through the cellular membrane on mitochondria to increase the intracellular level of calcium and generation of reactive oxygen species. S14G-humanin blocks these responses on the membrane, and partially protects mitochondria to restore the perturbed cellular homeostasis. These findings present a novel concept into Alzheimer's disease treatment, and are of scientific significance in revealing the mechanism by which S14G-humanin protects neurons.

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(Reviewed by Farso M, Raye W, Xiong K, Yu M)
(Edited by Mu WJ, Li CH, Song LP, Liu WJ, Zhao M)