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Berberine alters the processing of Alzheimer's amyloid precursor protein to decrease Aβ secretion

Masashi Asai [a,b,*], Nobuhisa Iwata [b], Ayumu Yoshikawa [a], Yoshimi Aizaki [a], Shoichi Ishiura [c], Takaomi C. Saido [b], Kei Maruyama [a,*]

[a] *Department of Pharmacology, Faculty of Medicine, Saitama Medical University, 38 Moro-hongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan*
[b] *Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Japan*
[c] *Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Japan*

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Abstract

Berberine is an isoquinoline alkaloid isolated from *Coptidis rhizoma*, a major herb widely used in Chinese herbal medicine. Berberine's biological activity includes antidiarrheal, antimicrobial, and anti-inflammatory effects. Recent findings show that berberine prevents neuronal damage due to ischemia or oxidative stress and that it might act as a novel cholesterol-lowering compound. The accumulation of amyloid-β peptide (Aβ) derived from amyloid precursor protein (APP) is a triggering event leading to the pathological cascade of Alzheimer's disease (AD); therefore the inhibition of Aβ production should be a rational therapeutic strategy in the prevention and treatment of AD. Here, we report that berberine reduces Aβ levels by modulating APP processing in human neuroglioma H4 cells stably expressing Swedish-type of APP at the range of berberine concentration without cellular toxicity. Our results indicate that berberine would be a promising candidate for the treatment of AD.

Keywords: Alzheimer's disease; Amyloid-β peptide; Amyloid precursor protein; Berberine; Cholesterol; Secretase

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common cause of dementia in the elderly [1,2]. Growing evidence suggests that amyloid-β peptide (Aβ) plays a critical and primary role in the pathogenesis of AD. The aggregation and accumulation of extracellular Aβ in the brain is a triggering event leading to the pathological amyloid cascade that typifies AD, including the formation of intracellular neurofibrillary tangles and the loss of synapses and neurons [1–4]. Aβ is generated by β- and γ-secretase-mediated sequential cleavages of amyloid precursor protein (APP), an integral membrane protein [1,2]. β-Secretase, a transmembrane aspartic protease known as BACE (beta-site APP cleaving enzyme) [1,5], cleaves at the N-terminus of the Aβ domain in APP, releasing its large soluble extracellular fragment (sAPPβ) and a membrane-anchored C-terminal fragment of APP, C99 (also known as CTFβ). C99 undergoes the next cleavage at several positions within the transmembrane domain of APP by the γ-secretase presenilin complex [6–8]; then $Aβ_{40}$ or $Aβ_{42}$ is generated. Alternatively, the third enzyme, called α-secretase, member of the ADAM (a disintegrin and metalloprotease) family, cleaves APP within the Aβ domain, thereby precluding production of Aβ [1,2,9]. Because the generation of Aβ is the rate-limiting step in the Alzheimer's amyloid cascade [1–3,10,11], the

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; APP_{NL}-H4 cells, H4 cells stably expressing Swedish-type of human APP_{695}; ELISA, enzyme-linked immunosorbent assay; FL, full-length; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; sAPP, soluble extracellular fragment of APP; sAPPα, sAPP generated by α-secretase; sAPPβ, sAPP generated by β-secretase.

* Corresponding authors. Fax: +81 49 276 1585.
E-mail addresses: asai@saitama-med.ac.jp (M. Asai), keimaru@saitama-med.ac.jp (K. Maruyama).

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modulation of APP metabolism would be a useful therapeutic strategy for the treatment of AD [12,13].

Recent results indicate that the amyloidogenic processing of APP occurs primarily in lipid rafts, which are specialized membrane microdomains rich in cholesterol, glycosphingolipids, sphingomyelin, and acylated proteins. It has also been found that Aβ production is sensitive to changes in cholesterol levels [14,15]. Since treatment with the cholesterol-lowering drugs known as statins—widely used inhibitors of cholesterol synthesis—causes both down-regulation of β-secretase activity [16] and upregulation of α-secretase activity [17], cholesterol-lowering drugs have great potential for the treatment of AD [14,18,19].

Berberine (benzyltetrahydroxyquinoline) is an isoquinoline-type alkaloid isolated from the Chinese herb *Coptidis rhizoma*; it has been widely used as a Chinese herbal medication. Because berberine has multiple pharmacological activities—including antidiarrheal, antimicrobial, and anti-inflammatory effects [20–22]—it has been used for centuries in the treatment of liver disease, skin inflammation, diarrhea, and other disorders. Recently, berberine has been also characterized as a novel cholesterol-lowering drug with a mechanism of action differing from that of the statins [23].

In this study, we focused on the cholesterol-lowering effect of berberine and attempted to investigate its possible effects on APP metabolism and Aβ levels in human neuroglioma H4 cells stably expressing Swedish-type of APP_{695} (APP_{NL}-H4 cells).

Materials and methods

Reagents. Berberine chloride was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA) and after reconstitution in distilled water, sterilized using a 0.22-μm filter. Hydrogen peroxide was purchased from Santoku Chemical Industries Co., Ltd. (Tokyo, Japan) and diluted with distilled water.

Cell culture. H4 neuroglioma cells (APP_{NL}-H4 cells) that stably express Swedish-type mutated human APP_{695} (APP_{NL} mutation, Lys595Asn, Met596Leu; expression vector pCEP4) (Invitrogen Japan K.K., Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37 °C with 5% CO_2. The medium was supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen Japan K.K.), and 150 μg/mL hygromycin B (Wako Pure Chemical Industries, Osaka, Japan).

Cellular toxicity analysis. APP_{NL}-H4 cells were plated at a density of approximately 1×10^4 cells per well on 24-well plates. After 24 h of incubation, the conditioned media were replaced with new media containing berberine at the final concentration indicated. Forty-eight hours after replacement of the media, lactate dehydrogenase (LDH) activity was determined to evaluate the cell toxicity of berberine by using the Cytotoxicity Detection Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Hydrogen peroxide was used as a positive control and added to the conditioned media during the last hour of incubation. The baseline was determined in control wells containing no cells and the obtained values were subtracted.

Sandwich ELISA. APP_{NL}-H4 cells were plated at a density of approximately 4×10^4 cells per well on 6-well plates. After 24 h of incubation, the conditioned media were replaced by new media containing berberine at the final concentration indicated. Forty-eight hours after replacement of the media, the cultured media were harvested and extracellular Aβ levels determined by using the Human Amyloid β Assay Kit (L)—IBL (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), according to the manufacturer's instructions.

Western blot analysis. APP_{NL}-H4 cells were plated at a density of approximately 4×10^4 cells per well on 6-well plates. After 24 h of incubation, the conditioned media were replaced by new media containing berberine at the final concentration of 1 μM. Forty-eight hours after replacement of the media, the cultured media were harvested and concentrated with trichloroacetic acid. The concentrates were then resuspended in a sample buffer containing β-mercaptoethanol and subjected to SDS–PAGE. The levels of sAPP (total soluble extracellular fragments of APP), sAPPα (sAPP generated by α-secretase), and sAPPβ (sAPP generated by β-secretase) in the media were quantified by Western blot analysis using monoclonal antibodies 22C11 (1:1000 dilution, amino acids 66–81 of the N terminus of APP, Chemicon International Inc., Temecula, CA, USA), 6E10 (1:500 dilution, amino acids 1–17 of the N terminus of Aβ, Signet Laboratories Inc., Dedham, MA, USA), and polyclonal anti-$sAPP\beta_{NL}$ antibody, produced in rabbits by injecting synthetic peptides, which is specific to soluble N-terminal fragments of APP with Swedish mutation, respectively. Cell lysates were also suspended in the sample buffer and subjected to SDS–PAGE. The levels of full-length APP (FL-APP) and β-actin in the cell lysates were quantified by Western blot analysis using polyclonal anti-APP C-terminal antibody A8717 (1:15,000 dilution, Sigma–Aldrich Inc.) and monoclonal anti-β-actin antibody AC-74 (1:5000 dilution, Sigma–Aldrich Inc.), respectively. Following application of peroxidase-conjugated secondary antibodies (Amersham Biosciences K.K., Tokyo, Japan), immunoreactive signals were detected by enhanced chemiluminescence using ECL Plus Western blotting detection reagents (Amersham Biosciences K.K.); signal intensity was determined with a densitometer, LAS-3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan), using Science Lab 2001 Image Gauge software (version 4.0; Fuji Photo Film Co., Ltd.). The amounts of immunoreactive soluble APP fragments, FL-APP, and β-actin in each sample were calculated based on standard curves constructed with one of the samples.

Statistical analysis. All values were expressed as the means ± SD. For comparison of two groups, a two-tailed Student's *t*-test was used. For comparison among more than three groups, Dunnett's multiple comparison test was used. Differences were considered significant if the *P* value was less than 0.05.

Results

Effect of berberine on cell viability

We first evaluated the cytotoxic effect of berberine in APP_{NL}-H4 cells. Compared with vehicle treatment, berberine showed no significant effect on LDH release into the culture medium between 0.1 and 100 μM for 48 h of incubation (Fig. 1). Compared with vehicle treatment, hydrogen peroxide used as a positive control strongly leads to cell death (262 ± 17%, $P < 0.01$) (Fig. 1). Berberine also had no effect on multiplication rate or the cell morphology of APP_{NL}-H4 cells through several passages for 4 weeks (data not shown).

Reduction in Aβ levels by berberine

We next examined extracellular Aβ levels in the cultured medium from APP_{NL}-H4 cells by sandwich ELISA (Fig. 2). Berberine treatment effectively reduced levels of $A\beta_{40}$ (47.1 ± 11.5% at 5 μM; 35.5 ± 8.1% at 50 μM) and $A\beta_{42}$ (49.1 ± 13.6% at 5 μM; 35.3 ± 10.2% at 50 μM); its 50% inhibition concentration (IC_{50}) for extracellular Aβ

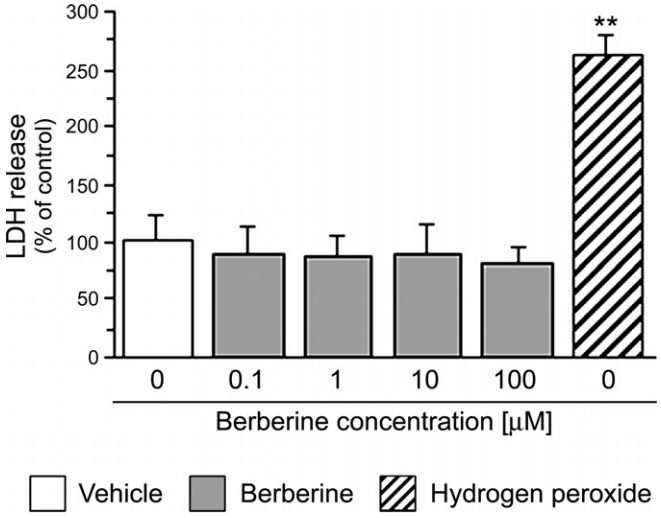

Fig. 1. Effect of berberine on cell toxicity in APP_{NL}-H4 cells. Amount of LDH released into the conditioned media from APP_{NL}-H4 cells treated with/without berberine was measured. Hydrogen peroxide was used as a positive control. Columns with bar represent the means ± SD of five independent experiments. $^{**}P < 0.01$, significantly different from the vehicle-treated group.

production was around 5 μM (Fig. 2a and b). It has recently been reported that some nonsteroidal anti-inflammatory drugs (NSAIDs) preferentially decrease only the level of $A\beta_{42}$ in cultured cells without significantly affecting APP processing by α- and β-secretases or Notch cleavage by γ-secretase [24]. However, berberine, unlike NSAIDs, did not show any significant change in the ratio of $A\beta_{42}/A\beta_{40}$ secretion (Fig. 2c).

Alteration of APP processing by berberine

Finally, we assessed the effect of berberine on α- and/or β-secretase activities in APP_{NL}-H4 cells by quantitative Western blotting (Fig. 3). Berberine treatment did not show significant changes in levels of either APP expression in APP_{NL}-H4 cells (99.3 ± 7.0%, $P > 0.9$) (Fig. 3b and c) or sAPP secreted into the media (103.9 ± 12.5%, $P > 0.7$) (Fig. 3b and d). In contrast, berberine stimulated α-secretase activity (increase = 311.9 ± 7.9%, $P < 0.01$) (Fig. 3b and e), and downregulated β-secretase activity (decrease = 55.5 ± 1.1%, $P < 0.01$) (Fig. 3b and f) in APP_{NL}-H4 cells. Thus, berberine modulated APP metabolism and shifted from an amyloidogenic to a nonamyloidogenic pathway.

Discussion

Accumulating epidemiologic and biochemical data, as reviewed by Simons et al. [18] and Puglielli et al. [19], suggest a relationship between cholesterol level and the pathogenesis of AD. For instance, apolipoprotein E (ApoE), a protein component of very low-density lipoprotein

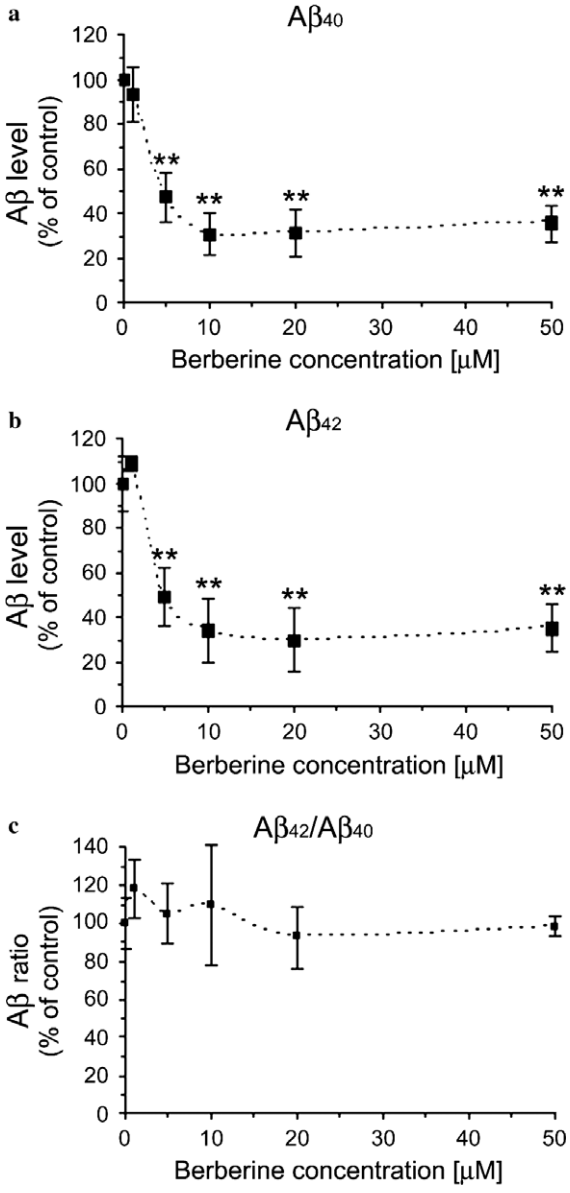

Fig. 2. Reduction of Aβ production by berberine in APP_{NL}-H4 cells. Amounts of Aβ released into the conditioned medium from APP_{NL}-H4 cells treated with/without berberine were measured by sandwich ELISA. Levels and ratio of Aβ were expressed as $A\beta_{40}$ (a), $A\beta_{42}$ (b), and $A\beta_{42}/A\beta_{40}$ (c), respectively. Data represent the means ± SD of four independent experiments. $^{**}P < 0.01$, significantly different from the vehicle-treated group.

(VLDL), is the major apolipoprotein in the brain, and incidence of the *APOE* ε4 allele is well-known as a genetic risk factor associated with late-onset AD [19]. Because high dietary levels of cholesterol increased the accumulation of Aβ in the brains of AD-model mice [25], treatment of AD with cholesterol-lowering drugs is of major interest to numerous research groups [17,26–28].

Statins—3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors—such as lovastatin and acyl coenzyme A cholesterol acyltransferase (ACAT) inhibitors, respectively, caused a significant increase in the

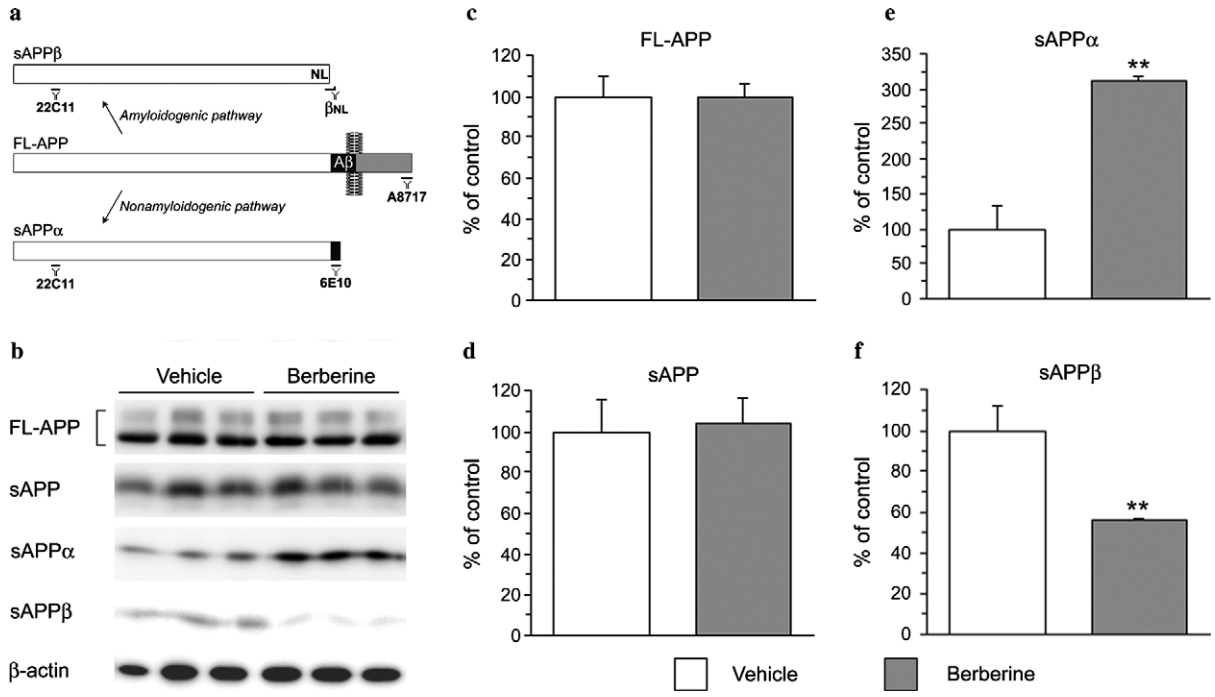

Fig. 3. Alteration in APP processing by berberine in APP_{NL}-H4 cells. Recognition sites of anti-APP antibodies used for quantitative Western blot analysis are shown in (a). Amounts of APP fragments and β-actin from APP_{NL}-H4 cells treated with/without berberine were analyzed by quantitative Western blot analysis. The typical blots of FL-APP, sAPP, sAPPα, sAPPβ, and β-actin are shown in (b). Levels of APP fragments were expressed as FL-APP (c), sAPP (d), sAPPα (e), and sAPPβ (f), respectively. Columns with bar represent the means ± SD of three independent experiments. $^{**}P < 0.01$, significantly different from the vehicle-treated group.

amount of sAPPα [17] and decrease in Aβ levels by decreasing cholesterol level in H4 cells [27]. Berberine stabilized low-density lipoprotein (LDL) receptor mRNA and upregulated LDL receptor expression, resulting in the reduction of serum cholesterol, triglycerides, and LDL cholesterol [23]. Because LDL receptor mRNA is indeed expressed in APP_{NL}-H4 cells (data not shown), berberine may act as a cholesterol-lowering drug in this experimental paradigm and may decrease extracellular Aβ levels by modulating APP processing. However, it remains possible that berberine, irrespective of cholesterol metabolism, may decrease Aβ levels in cultured cells [17,26,27,29] because it has other significant activities, such as acetylcholinesterase inhibitory activity [30]. Acetylcholinesterase inhibitors are currently the only available drugs for AD treatment. Donepezil hydrochloride (Aricept), the most specific and potent acetylcholinesterase inhibitor, not only leads to enhancement of cholinergic activity in the brains of patients with amnestic mild cognitive impairment but is also capable of promoting the translocation of an α-secretase candidate ADAM10 to the cell surface [31,32]. The latter would result in a nonamyloidogenic effect via the upregulation of α-secretase activity [32]. Therefore, the nonamyloidogenic effect of berberine may be associated with its acetylcholinesterase inhibitory activity.

Recent studies have indicated that particular forms of soluble Aβ, such as oligomeric forms, cause toxicity to neurons [2,3]. Since APP_{NL}-H4 cells overproducing Aβ grow as much as wild ones do, H4 cells are fairly resistant to its toxicity or it might be possible that the oligomerization of Aβ be somehow disturbed in culture media. Whether or not the berberine protects neurons from Aβ toxicity will be examined in future, for example, in transgenic mice with senile plaques.

It has been reported that berberine given orally effectively lowers serum cholesterol and LDL cholesterol in hyperlipidemic hamsters and human hypercholesterolemic patients [23]. In addition, it has been shown that berberine, a quaternary ammonium salt, can pass through the blood–brain barrier [33] and reach the brain parenchyma in a dose- and time-dependent manner [34]. Thus berberine would be a promising Aβ-lowering drug to be given orally for the treatment of AD.

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