Formononetin Protects Neurons Against Hypoxia-Induced Cytotoxicity Through Upregulation of ADAM10 and sAβPPα

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Abstract. Formononetin, an active constituent of the Chinese herb Astragalus Radix, has been reported to have beneficial effects for Alzheimer’s disease (AD). Yet the mechanism of this effect remains to be elucidated. The present study shows that formononetin increases soluble-AβPPs (sAβPPs) secretion and thus protects human-AβPP Swedish mutation cell (N2a-AβPP cell) from hypoxia-induced apoptosis. Using hypoxic N2a-AβPP cell as an in vitro model of AD-like pathology, we confirmed that regular treatment with formononetin could have neuroprotective effects, followed respectively by reduced caspase 3 activity and increased cell viability. Strikingly, our data revealed that the caspase 3-blocking effect of formononetin was largely mediated by stimulation of Aβ-secretase cleavage of AβPP, and increasing the secretion of its soluble form, sAβPPs. Moreover, the protective effect of formononetin was totally inhibited by TAPI-2, an Aβ-secretase complex inhibitor, suggesting the role of the sAβPPs pathway in the neuroprotective response to formononetin. We also found that the stimulative effect of formononetin on Aβ-secretase activity was mainly conducted by upregulating ADAM10 expression at the transcriptional level. Altogether, our study provides novel insights into how formononetin mediates stimulation of the ADAM10-sAβPPs pathway and exerts a neuronal protective effect.

Keywords: ADAM10, amyloid-β protein precursor (AβPP), formononetin, soluble-AβPP

Supplementary data available online: http://www.j-alz.com/issues/28/vol28-4.html#supplementarydata03

INTRODUCTION

Formononetin [7-hydroxy-3-(4-methoxyphenyl)chromone or 4′-methoxy daidzein] is a soy isoflavonoid found abundantly in traditional Chinese medicine Astragalus mongholicus (Bunge) and Trifolium pretense L. (red clover). It belongs to the phytoestrogens due to its similar chemical structure to gonadal steroid estrogen [1]. Studies have found
that formononetin can promote endothelial repair and wound healing [2], prevent lipopolysaccharide-induced injury in dopaminergic neurons [3], and protect PC12 cells as well as cultured cortical and hippocampal neurons from oxidative stress and toxicity induced by L-glutamate or amyloid-β [4–7], suggesting a neuroprotective effect of formononetin.

The soluble fragment of the amyloid-β protein precursor (sAβPPs) is the product of AβPP cleavage by α-secretase in the non-amyloidogenic pathway. It has been suggested that decreased sAβPPs correlates with memory loss during aging in a rat model [8], and generation of sAβPPs has been found to be down-regulated in aging human fibroblasts [9]. Moreover, sAβPPs formation tends to be down-regulated in response to ischemia [10, 11]. In neuronal precursors and non-neuronal cells, sAβPPs acts as a proliferative factor [12–14], involved in both cell differentiation induction [15] and neurite outgrowth [16]. Besides these proliferative effects, sAβPPs also plays an important role in neuronal plasticity and memory [17, 18]. Furthermore, sAβPPs has been found to have a neuroprotective effect against cell apoptosis [20] and to improve the outcome of severe brain injury [19]. Therefore upregulating sAβPPs could be a potential target for AD treatment, and to find out whether and how formononetin could regulate AβPP processing and sAβPPs formation would be of great interest [21].

Here, we used a mouse neuroblastoma cell line, stably transfected with human-AβPP Swedish mutation (N2a-AβPP) to evaluate the effects of formononetin on hypoxia-induced reduction of sAβPPs secretion and subsequent neuron injury. Results of the study provide a novel neuroprotective mechanism of formononetin mediated by increasing sAβPPs secretion and α-secretase and A-Disintegrin-And-Metalloproteinase (ADAM) 10 expression.

MATERIALS AND METHODS

Formononetin extraction

Astragalus Radix was collected from Hunyuan County, Shanxi Province of China, and authenticated by Professor Shao-Qing CAI as the roots of Astragalus membranaceus (Fisch.) Bge. var. mongholicus 11/4/2011(Bge.) Hsiao. Formononetin was isolated from Astragalus Radix by chromatographic methods, including column chromatography on D-101 macroporous adsorption resin, silica gel, and was identified on the basis of spectral data, including UV, 1H-NMR, and 13C-MNR. The purity of formononetin by HPLC-UV analysis was above 95% (Fig. 1).

Reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), and N,N-dimethyl sulfoxide (DMSO), were purchased from Sigma (St. Louis, MO, USA). The α-secretase inhibitor TAPI-2 was purchased from Calbiochem (San Diego, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). The EDH kit was purchased from Promega (Madison, WI, USA), and the sAβPPa activity kit was purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture and hypoxia treatments

N2a cells stably transfected with human-AβPP95sw (N2a-AβPP) were grown in DMEM supplemented with 10% FBS, at 37°C in an atmosphere of 5% CO2. For formononetin and hypoxia treatment, cells were pre-treated with formononetin for 4 h and then transferred into an anaerobic chamber (model 1029, Forma Scientific, Marietta, OH, USA, [21]) containing 85% N2/10% H2/5% CO2 for various durations.

Cell viability assay

MTT assay was carried out as previously described [22]. Cells were plated in 96-well plates, and the cell viability was determined by the conventional MTT reduction assay. The cells were exposed to hypoxic condition at different time points with/without various concentrations of formononetin for the indicated time. After incubation, cells were treated with the MTT solution (0.5 mg/ml) for 4 h at 37°C in the dark. 200 μl DMSO was added to the culture medium and mixed with a pipette until the blue formazan dissolved completely. The optical density of formazan was measured at 570 nm using a micro plated reader.
Flow cytometry

As the key index for apoptosis detection, Annexin V-FITC staining was performed to measure phosphatidylserine externalization in N2A-APP cells. Briefly, cells were processed with different treatments, then washed twice with PBS and resuspended in 200 μL of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 2.5 mM CaCl₂), 10 μL of Annexin V was then added to give a final concentration of 0.5 μg/mL. The staining sample was incubated at room temperature for 20 min, with out light. Subsequently, 5 μL of propidium iodine was added to the samples (final concentration of 1 μg/mL) and 10,000 cells were immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Results were calculated as a percentage of apoptotic cells.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme present in most eukaryotic cells, released into the culture medium upon cell death due to the damage of plasma membrane. The increase in LDH activity in the culture supernatant is proportional to the number of lysed cells. After cells were exposed to hypoxia, the supernatant and cell lysates were collected, and the amount of LDH release was determined using a standard kit according to the manufacturer’s protocol. Briefly, the supernatant and cell lysates were transferred to 96-well plates and incubated with 1 mM NADH in pyruvate substrate solution at 37°C for 20 min. After additional incubation at 37°C for 15 min with 2, 4-dinitrophenylhydrazine, the reaction was stopped by addition of 0.4 M NaOH. The changes in absorbance were determined at 440 nm using a spectrophotometer microplate reader. LDH leakage was expressed as the percentage (%) of the total LDH activity (LDH in the supernatant + LDH in the cell lysate), according to the equation: %LDH released = (LDH activity in the supernatant + LDH activity in the cell lysate) / total LDH activity × 100%.

α-secretase activity measurement

α-secretase activity was measured following the manufacturer’s instructions (GBD, San Diego, CA, USA). Cells treated with formononetin in the presence or absence of hypoxia treatment were allowed to swell by adding 100 μL of PBS and resuspended in 200 μL of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 2.5 mM CaCl₂). 10 μL of formononetin was then added to give a final concentration of 0.5 μg/mL. The staining sample was incubated at room temperature for 20 min, with out light. Subsequently, 5 μL of propidium iodine was added to the samples (final concentration of 1 μg/mL) and 10,000 cells were immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Results were calculated as a percentage of apoptotic cells.
proteins were transferred onto PVDF membranes, subsequently immunostained with the following primary antibodies against ADAM9 (1 : 500, Sigma), ADAM10 (1 : 2000, Abcam), ADAM17 (1 : 500, Sigma), and GAPDH (1 : 5000, Calbiochem). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1 : 20000) for 1 h at ambient temperature, the immunoblots were developed using the ECL system.

Quantitative densitometric analyses were performed on digitized images of immunoblots with Quantity One software (Bio-Rad, Hercules, CA, USA). Representative blots from at least three independent experiments were shown.

Measurement of caspase-3 activity

Caspase activity was assayed using caspase-3 activity assay kit (MBL) according to the manufacturer’s instructions. Briefly, cells were collected and washed with PBS, then resuspended in a cell lysis buffer. After incubation on ice for 10 min, the lysates were centrifuged for 20 min at 12,000 × g, and the supernatants were collected and protein concentrations were determined. Cell lysates (100 μg) were mixed with reaction buffer containing the DEVD-pNA substrate (200 μM) for caspase 3 activity. The absorbance was measured in the wells at 405 nm using an ELISA reader.

RT-PCR measurement

To detect mRNA levels, total RNA was extracted with TRIZOL (Invitrogen), and converted to cDNA by reverse kit (Takara) according to the manufacturer’s instructions. ADAM10 sense, primer 5′-TGTACGCCTCTGGCCG3′; ADAM10 antisense primer, 5′-GGTATATGCGCCATACACTACCC-3′; b-actin sense primer, 5′-TGTAACCTCTGGCCG3′; b-actin antisense primer, 5′-CCACGTCACCTCATTGGG3′. PCR reactions were performed at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min during 40 cycles, followed by a final extension of 7 min at 68°C. The resulting PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

siRNA-mediated knockdown

The siRNA method was performed according to previous work [48]. N2A-AβPP cells were reverse transfected with 20 nM small interfering RNA (siRNA) duplexes by the use of Lipofectamine RNA inter-
had a protective effect similar with that of EGb (Fig. 2C). Accordingly, we also evaluated LDH activity in culture medium as an indicator of cell damage and showed that 10 µM formononetin pretreatment reduced hypoxia-mediated LDH release by nearly 40%, which further confirmed its protective role (Fig. 2D). As caspase-3 activity is an indicator of apoptosis [28], we measured its level under hypoxia in the presence or absence of different chemicals. In agreement with a previous report [29], we also observed a significant increase of caspase-3 activity after hypoxia treatment; meanwhile in formononetin pretreatment groups, we found that the caspase-3 activity showed a significant decrease compared with the levels of those without formononetin pretreatment (Fig. 2E).

Formononetin could stimulate α-secretase activity

Previous reports have shown that hypoxia treatment would induce a calcium influx, activate down-stream
M. Sun et al. / Formononetin Increases sAβPPβ and ADAM10 Level

Formononetin increases α-secretase activity by upregulating ADAM10 protein level, but not ADAM9 or ADAM17

As we have found formononetin treatment increases α-secretase activity, we further investigated its effect...
Fig. 4. Formononetin (FRM) could increase α-secretase by upregulating ADAM10 mRNA level. N2a-APP cells were pre-treated with or without FRM for 4 h and then hypoxia treatment for an additional 18 h. After that, cell lysates were collected for different measurements as follows: (A) α-secretase activity; (B) pre-mature form of ADAM10 protein level; (C) mature form of ADAM10 protein level; (D) The ratio of mature form of ADAM10 versus pre-mature form of ADAM10; (E) ADAM 9 protein level; and (F) ADAM17 protein level. Total mRNA was also isolated for detecting (G) ADAM10 mRNA level. All data were represented as a mean ± S.D. from triplicate independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
on the components of a-secretase complex to clarify the underlying mechanism. As shown in Fig. 4B and C, we found that the levels of both pre-mature and mature ADAM10 were decreased under hypoxia treatment and that formononetin treatment could significantly attenuate this decrease, as well as increase the mature/immature ratio of it compared with the hypoxia group (Fig. 4D). Meanwhile, other potential a-secretase candidates, ADAM9 and ADAM17, did not exhibit a statistically significant change under different experimental treatments (Fig. 4E, F).

Since our results showed that not only the mature form of ADAM10 was increased by formononetin treatment but also the pre-mature form, a question was raised regarding whether or not formononetin treatment could increase ADAM10 at the transcriptional level. As shown in Fig. 4G, employing conventional PCR, we found that the mRNA level of ADAM10 was significantly higher in the formononetin treatment group compared to the hypoxia group, suggesting that under hypoxic condition, formononetin treatment could recover ADAM10 at the transcriptional level.

**Formononetin’s protective effect is mainly dependent on the aβPPα pathway**

Since we found that formononetin could upregulate ADAM10 under hypoxic condition, followed by increasing aβPPα secretion levels, we next wondered whether formononetin’s effect on ADAM10 expression was hypoxia dependent. As shown in Fig. 5, we found under normoxia condition, when N2a-aβPP cells were treated with formononetin, the total protein level of ADAM10 and its mRNA level were both increased (Fig. 5C, D, lane 1 compared with lane 2). Furthermore, in addition to increasing ADAM10, formononetin administration could also upregulate a-secretase activity (Fig. 5B, lane 1 and lane 2), which indicated that formononetin could increase aβPPs by elevating a-secretase activity and ADAM10 transcriptional levels independent of hypoxia.

At last, we wanted to confirm whether the cell protective effect under hypoxic conditions was conducted by the ADAM10-aβPPα pathway activated by formononetin treatment. For this purpose, we used the a-secretase specific inhibitor, TAPI-2, which could

![Image](https://via.placeholder.com/150)

**Fig. 5. aβPPα secretion and a-secretase activity were totally abolished by TAPI-2.** A) N2a-aβPP cells were treated with formononetin (FRM) in the presence or absence of TAPI-2 for 24 h and then cells lysates were collected for different measurements as follows: (A) aβPPα protein level; (B) a-secretase activity; and (C) ADAM10 protein level. Total mRNA was also isolated for detecting (D) ADAM10 mRNA level. All data were represented as a mean ± S.D. from triplicate independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
significantly inhibit α-secretase activity and further decrease sAβPPs production (Fig. 5A, B) without influencing ADAM10 expression (Fig. 5C, D), and ADAM10 siRNA which could inhibit sAβPPs secretion while diminish ADAM10 expression (Fig. 7A, B, C). Treating the cells with TAPI-2 and formononetin for a total of 6 h followed by an additional 18 h of hypoxia treatment, we found while the sAβPPs secretion and α-secretase activity were almost totally abolished (Fig. 6A, B), the mRNA level of ADAM10 was hardly influenced by TAPI-2 under hypoxia treatment (Fig. 6C). Meanwhile both MTT and flow cytometry assays showed a decrease of cell viability in formononetin with the TAPI-2 group compared with the formononetin alone group under hypoxic conditions (Fig. 6D–I). Furthermore, ADAM10 siRNA treatment also appeared to give a similar result. As shown in Fig. 7D–J, flow cytometry assay showed a nearly 40% decrease of living cell number in the hypoxia group compared with control, and formononetin treatment could attenuate hypoxia-induced cell impairment, while ADAM10 siRNA could almost

Fig. 6. The neuroprotective effect of formononetin (FRM) was inhibited by TAPI-2. N2a-AβPP cells were administrated with hypoxia treatment in the presence or absence of pre-treatment of FRM and TAPI-2. 18 h later, cells lysates or total mRNA was collected for different measurements as follows: (A) sAβPPs protein level; (B) α-secretase activity; (C) ADAM10 mRNA level. After 18 h hypoxia treatment, cells were also collected for detecting apoptosis rate or cell viability. D–H) Flow cytometry assay for neuro-apoptosis rate measurement in the presence or absence of FRM or TAPI-2. 1) Viability of N2a-AβPP cell after 18 h hypoxia treatment with FRM and TAPI-2 was measured by MTT assay. All data were represented as a mean ± S.D. from triplicate independent experiments. *p<0.05, **p<0.01, ***p<0.001.
Fig. 7. The neuroprotective effect of formononetin (FRM) was inhibited by ADAM10 siRNA. N2a-sAPP cells were transfected by ADAM10 siRNA for 24 h, and then (A) ADAM10 protein level; (B) α-secretase activity; and (C) ADAM10 mRNA level was detected. Cells were exposed to hypoxia in the presence or absence of FRM pre-treatment for 18 h, and then cells were collected for different measurements as follows: D–H) flow cytometry assay for the apoptosis rate measurement in the presence or absence of FRM and ADAM10 siRNA. I) MTT assay for cell viability measurement after 18 h hypoxia treatment with FRM and ADAM10 siRNA. J) Caspase-3 activity after 18 h hypoxia treatment with FRM and ADAM10 siRNA was measured. All data were represented as a mean ± S.D. from triplicate independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

abolish formononetin-induced protective effects and reduction in caspase-3 activity. MTT assay also showed a similar result (Fig. 7I). All results above indicated that the protective effect of formononetin against hypoxia was mainly conducted by the ADAM10-sAPP pathway.

**DISCUSSION**

In the AβPP non-amyloid cleavage pathway, AβPP would be cleaved by α-secretase and thus produce the neuroprotective fragment sAβPPα [35, 43]. Induced sAβPPα secretion might have additional advan-
tages, for various studies have strongly established that secreted s\(\beta\)PPs possesses potent neurotrophic and neuroprotective activities against excitotoxic and oxidative assaults [32, 36], INK-mediated apoptosis [37], and the pro-apoptotic action of mutant presenilin-1 by activating the transcription factor NF-\(\kappa\)B [38]. Moreover, s\(\beta\)PPs stimulates neurite outgrowth [39], regulates synaptogenesis [40], has trophic effects on cerebral neurons in culture [41], stabilizes neuronal calcium homeostasis, and protects hippocampal and cortical neurons against the toxic effects of glutamate and A\(\beta\) peptides [42]. Also, it has been shown that intracerebroventricular administration of secreted forms of s\(\beta\)PPs to amnestic mice had potent memory-enhancing effects and blocked learning deficits induced by scopolamine [18]. Therefore, increased production of s\(\beta\)PPs protein by upregulating ADAMs would potentially be beneficial for AD [44, 50].

Cerebral hypoxia results from an insufficient oxygen supply to the brain and causes neuronal damage in vulnerable brain areas [60]. Accumulating evidence indicates that cerebral hypoxia/stroke significantly increases AD risk [51-63]. Multiple studies have confirmed that hypoxia treatment could cause cell damage [review see 68], one of the mechanisms being the activation of caspase-3. Caspase-3 is the most abundant of the known caspases in neural cells, and appears to play a crucial role both during normal development and in situations of hypoxic injury [64]. It has been found that under hypoxic conditions, caspase-3 activity is stimulated by increased calcium influx, which is the common event after exposure to hypoxia [65, 66]. As s\(\beta\)PPs is also known to be a potent mediator attenuating excessive calcium entry and excitotoxicity [32, 67], we found formononetin’s inhibition effects on caspase-3 activity was mainly conducted by increasing s\(\beta\)PPs secretion level. Administration of ADAM10 siRNA, which would further inhibit s\(\beta\)PPs secretion and CTF-\(\beta\)PP level (supplementary Figure 1; available online: http://www.jalz.com/issues/28/v028-4.html#supplementarydata03) as well as a decrease of CTF-\(\alpha\).

Enzymes acting as \(\beta\)-secretase complex in the A\(\beta\)PP non-amyloid pathway have been identified as members of ADAM family. It has also been found that under hypoxic condition \(\alpha\)-secretase activity and s\(\beta\)PPs would decrease significantly [45, 56, 57]. Three of these membrane-anchored zinc-dependent metalloproteinases, ADAM10 as well as ADAM17 and ADAM9, are the candidate enzymes that show \(\alpha\)-secretase activity [47]. Among them, although ADAM10 has been extensively researched, the results are still controversial. Some studies found ADAM10 and TACE level significantly decreased after hypoxia treatment [34, 58], yet other studies, using primary cell of AD transgenic mice overexpressing mutant A\(\beta\)PP or cerebral microvascular smooth muscle cells as a model, found that ADAM10 was markedly increased in the early stage of ischemic insult or hypoxia treatment and afterwards decreased [55, 57]. Furthermore, another study found in the SH-SY5Y cell line that hypoxia treatment would change ADAM10 and BACE-1 levels but not ADAM10 or TACE [49]. While it is hard to reconcile these results, differing experimental procedures, such as cells/tissues examined, culture conditions, the percentage of \(O_2\) utilized, or duration of hypoxic treatment, could all contribute to discrepant results. In agreement with some previous works, we found a significant decrease in ADAM10 protein after 18 h of hypoxic treatment coincidently with a decrease in s\(\beta\)PPs secretion and CTF-\(\alpha\) level.

SIRT1 is one of the seven mammalian proteins of the sirtuin family of NAD (+)-dependent deacetylases, and its hyperactivity might be able to prevent AD pathology both in vitro and in vivo. It has been recently found that SIRT1 could directly activate transcription of ADAM10, elevating both mature and immature forms of ADAM10, but not ADAM9 or ADAM17, and further increasing the activity of \(\alpha\)-secretase [50]. Additionally, there was a study showing
that, as one of the chemicals belonging to isoflavone, formononetin could increase both the activity and expression of SIRT1 [51]. Combined with the aforementioned results, the fact that formononetin could increase SIRT1 expression and that SIRT1 had no effects on ADAM9 or ADAM17 gives us a clue that formononetin probably regulates ADAM10 by modulating SIRT1 level.

Here we report that after formononetin administration, the α-secretase activity of N2a-AβPP cells was significantly higher than the hypoxia treatment group. Our results also show that mature and immature forms of ADAM10 were elevated while ADAM9 and ADAM17 did not change. Interestingly, we also found that the ratio of ADAM10 mature to immature form was increased by formononetin treatment, which indicates that formononetin may not only regulate ADAM10 on a transcriptional level, but also influence ADAM10 protein trafficking and subcellular location.

In summary, we demonstrated for the first time that formononetin prevented hypoxia-induced neurotoxicity by increasing sAβPPα expression in N2a-AβPP cells, and we further found that the effect of formononetin on sAβPPα levels was mainly mediated by upregulating ADAM10 at the transcriptional level and further increasing α-secretase activity. These findings provide novel insights on formononetin’s neuroprotective mechanisms and the signaling pathways involved, and could be valuable to future therapeutic research.

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