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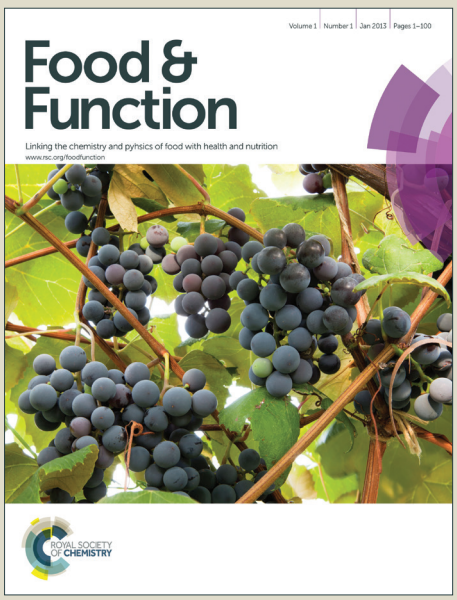

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Anti-apoptotic and anti-glycative effects of asiatic acid in brain of d-galactose treated mice

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Running title: anti-aging effects of asiatic acid

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Abstract

Protection of asiatic acid (AA) in mice brain against d-galactose (DG) induced aging was examined. AA at 5, 10 or 20 mg/kg/day was supplied to DG treated mice for 8 wks. AA intake at 10 or 20 mg/kg/day increased its deposit in brain. DG treatment increased Bax and cleaved caspase-3 protein expression, and decreased Bcl-2 expression. AA intake at 10 and 20 mg/kg/day declined Bax and cleaved caspase-3 expression, and retained Bcl-2 expression. DG treatment decreased brain glutathione content and glutathione peroxidase activity; increased brain reactive oxygen species and protein carbonyl levels, and enhanced NAPDH oxidase expression. AA intake at test doses reversed these changes. DG treatment up-regulated the expression of advanced glycation endproduct (AGE), carboxymethyllysine, receptor of AGE (RAGE), mitogen-activated protein kinases and CD11b; as well as increased interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha release in brain. AA intake at 5, 10 and 20 mg/kg/day lowered AGE and carboxymethyllysine expression, and at 10 and 20 mg/kg/day reduced RAGE production. AA intake dose-dependently suppressed p-p38 expression and lowered IL-6 and TNF-alpha levels; and at 10 and 20 mg/kg/day down-regulated p-JNK and CD11b expression. DG treatment declined brain-derived neurotropic factor (BDNF) expression and raised glial fibrillary acidic protein (GFAP) expression. AA intake at 20 mg/kg/day retained BDNF expression, and at 10 and 20 mg/kg/day reduced GFAP expression. These findings indicated that the supplement of asiatic acid might benefit the prevention or alleviation of brain aging.

Keywords: Aging; Asiatic acid; Glycation; NADPH oxidase; MAPK

Introduction

Apoptosis, oxidation and glycation play important roles in the pathological progression of aging and neurodegenerative diseases. Mitochondrial apoptotic pathway is involved in neurons death. It is mainly mediated by BCL family proteins, which include pro-apoptotic molecules such as Bax, and anti-apoptotic molecules such as Bcl-2 (Pollack et al. 2002). Caspase-3 is also responsible for neuro-apoptosis in brain tissue (Niu et al. 2010). Thus, any agent with the ability to regulate Bcl-2, Bax and caspase-3 may potentially attenuate nerve cell apoptosis and delay aging.

NADPH oxidase complex is a key regulator for reactive oxygen species (ROS) generation and involved in the progression of aging and cerebrovascular diseases (Chrissobolis and Faraci 2008). ROS overproduction activates mitogen-activated protein kinases (MAPK) pathway, which consequently exacerbates oxidative injury and promotes neuron death (Ralay et al. 2012). The presence of advanced glycation endproducts (AGEs) such as carboxymethyllysine (CML) in brain increases glycative stress, and has been considered as a feature of aging and degeneration (Münch et al. 1997). AGEs could up-regulate the receptor for AGEs (RAGE), and the engagement of AGEs-RAGE could also activate MAPK, and enhance the generation of inflammatory cytokines such as tumor necrosis factor (TNF)-alpha (Younessi and Yoonessi 2011). Thus, the agent with the effects to lower brain ROS and AGEs production; and to decline brain RAGE and MAPK expression may diminish glycative stress and restrict aging progression. In addition, brain-derived neurotropic factor (BDNF) is a regulator for synaptic formation of central and peripheral neurons (Dwivedi 2013). Glial fibrillary acidic protein (GFAP) is a marker of astrogliosis; and its overproduction is highly associated with cognitive impairment (Jin et al. 2014). Thus,

an agent may retard brain aging if it increases BDNF and/or decreases GFAP.

Asiatic acid is a pentacyclic triterpene naturally occurring in many vegetables and fruits such as basil (*Ocimum basilicum*), brown mustard (*Brassica juncea*) and centella (*Centella asiatica* L.) (Hashim et al. 2011; Yin et al. 2012). Krishnamurthy et al. (2009) reported that asiatic acid could decrease blood-brain barrier permeability and mitigate mitochondrial injury in a mouse model of focal cerebral ischemia. Xu et al. (2012) indicated that oral administration of asiatic acid at 100 mg/kg body weight improved brain oxidative stress and cognitive deficit in glutamate treated mice. Those previous studies revealed that this compound was a potent protective agent for brain. However, it remains unknown that asiatic acid could provide protection for brain against aging associated apoptotic and glycative injury.

D-galactose (DG)-induced neuro-pathological alteration has been used as an aging model because DG over-supply induces apoptotic, oxidative and glycative stress in the nervous system (Lu et al. 2010; Han et al. 2014). In our present study, DG injected mice were used to examine the brain protection of asiatic acid. The effects of this compound at various doses upon brain level of ROS, AGEs, CML and inflammatory cytokines were measured. The impact of this agent upon protein expression of Bcl-2, Bax, NAPDH oxidase, RAGE, MAPK and GFAP were determined in order to elucidate its possible action modes.

Materials and Methods

Materials

Asiatic acid (AA, 95%) and DG (99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used in these measurements were of the highest purity commercially available.

Animals and diet

Male Balb/cA mice, 3 wk old, were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12-h light:dark schedule; water and mouse standard diet were consumed *ad libitum*. The use of mice was reviewed and approved by China Medical University animal care and use committee (CMU-102-23-N).

Experimental design

Mice at 7-month old were used for experiments. Mice were divided into two groups, in which one group was treated with DG (100 mg/kg body weight) via daily subcutaneous injection for 8 weeks. Song et al. (1999) indicated that 8-wk DG injection induced 24 months aging. Thus, 8-wk DG injection for 7-month old mice caused 31-month old, which was approximately equal to 80-year-old human (Harrison, 2010). DG treated mice were further divided into four sub-groups, in which AA at 0, 5, 10 or 20 mg/kg/day was supplied. AA, suspending in 0.8% methyl cellulose, was administered daily by oral gavage. Our preliminary study revealed that methyl cellulose at this dose did not affect any measurements. Non-DG treated mice were divided into two sub-groups, in which AA at 0 (control) or 20 mg/kg/day was supplied. After 8-wk treatments, mice were sacrificed by decapitation. Brain was quickly removed, and at 0.1 g was homogenized on ice in 2 ml of phosphate buffer saline (PBS, pH 7.2). Protein concentration of brain homogenate was determined by a commercial assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin as a standard. In all experiments, sample was diluted to a final concentration of 1 mg protein/ml.

Brain AA Content

The content of AA in brain was analyzed by the method described in Gerbeth et al. (2011). Brain homogenate, 100 μl, was mixed with glycyrrhetinic acid as an internal standard (10 μl of 2.0 μg/ml methanol solution), and followed by extracting with 1 ml ethyl acetate and centrifuging at 3500 xg for 10 min at 4°C. After evaporated by nitrogen, the residue was reconstituted in 100 μl of methanol and water, the mobile phase of HPLC. Identification and quantification was processed by an HPLC-MS system (Agilent Corp, Waldbronn, Germany), in which Agilent 1100 series HPLC equipped with a BDS RP-C18 column (100 mm × 4 mm, 3 μm, Thermo Electron, Bellafonte, PA, USA), a diode array and a fluorescence detector was used. An ion-trap mass spectrometer equipped with an electro-spray ionization source was coupled with this HPLC. The limit of detection was 0.1 μg/g tissue.

Determination of ROS, protein carbonyl and glutathione (GSH) levels, and glutathione peroxidase (GPX) activity

Intracellular ROS level was determined using a oxidation sensitive dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, 100 μl homogenate was mixed with 100 μl of 2 mg/ml DCFH-DA for 30 min at 37°C. Fluorescence was measured at 488 nm excitation and 525 nm emission using a fluorescence plate reader. Results are expressed as relative fluorescence unit (RFU) per mg protein. Protein carbonyls were determined with the Zentech PC kit (BioCell, Auckland, New Zealand). Briefly, 50 μl sample was mixed with a 200 μl dinitrophenylhydrazine (DNP) solution. The adsorbed DNP–protein was reacted with an anti-DNP-biotin antibody, and followed by reacting with streptavidin-linked horseradish peroxidase probe and chromatin reagent. The absorbance at 450 nm was measured. The concentration of reduced GSH was determined by a commercial colorimetric GSH assay kit (OxisResearch, Portland, OR, USA). GPX activity (U/mg protein) was determined by an

assay kit (Calbiochem, EMD Biosciences, Inc. San Diego, CA, USA).

Measurement of interleukin (IL)-6 and TNF-alpha

Brain tissue was homogenized in 10 mM Tris-HCl buffered solution (pH 7.4) containing 2 M NaCl, 1 mM EDTA, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 9000 xg for 30 min at 4°C. The resultant supernatant was used for cytokine determination. The levels of IL-6 and TNF-alpha were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA, USA). The limit of detection was 5 nmol/l for IL-6, and 10 nmol/l for TNF-alpha.

Western blot analysis

Brain tissue was homogenized in buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). This homogenate was further mixed with buffer (60 mM Tris-HCl, 2% SDS, and 2% β-mercaptoethanol, pH 7.2), and boiled for 5 min. Sample at 40 μg protein was applied to 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) for 1 hr. After blocking with a solution containing 5% nonfat milk for 1 hr to prevent non-specific binding of antibody, membrane was incubated with mouse anti-cleaved caspase-3, anti-Bcl-2, anti-Bax (1:2000), anti-$p47^{phox}$, anti-$gp91^{phox}$ (1:1000), anti-RAGE, anti-CML, anti-AGE (1:500), anti-CD11b, anti-GFAP, anti-BDNF (1:1000) or anti-MAPK (1:2000) monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN, USA) at 4ºC overnight, and followed by reacting with horseradish peroxidase-conjugated antibody 3.5 hr at room temperature. The blot was imaged by autoradiography, and quantified by densitometric analysis. Results were normalized to GAPDH, and given as arbitrary units (AU).

Statistical analysis

The effect of each measurement was analyzed from 10 mice (n = 10). All data were expressed as mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance, and post-hoc comparisons were carried out using Dunnett's t-test. Statistical significance is defined as $p< 0.05$.

Results

AA treatments increased brain AA content

AA intake alone (without DG treatment) significantly increased brain AA content and GSH level (Tables 1 and 2, $p<0.05$), but did not affect other measurements when compared with control groups ($p>0.05$). As shown in Table 1, DG injection and AA treatments did not affect body weight, water intake, feed intake and brain weigh ($p>0.05$). Among all DG groups, AA intake at 10 and 20 mg/kg/day increased AA deposit in brain.

AA treatments attenuated brain apoptotic and oxidative stress

As shown in Figure 1, DG treatment increased 6.1 folds Bax and 6.5 folds cleaved caspase-3 protein expression, and decreased 89.2% Bcl-2 expression ($p<0.05$). AA intake at 10 and 20 mg/kg/day lowered 25.9-55.5% Bax and 46.4-48.1% cleaved caspase-3 protein expression; and restored 22.1-23.7% Bcl-2 expression when compared with DG treatment alone ($p<0.05$). DG treatment increased brain ROS and protein carbonyl levels, and decreased brain GSH content and GPX activity (Table 2, $p<0.05$). AA intake dose-dependently reduced ROS and protein carbonyl levels, and retained GSH level and GPX activity ($p<0.05$). As shown in Figure 2, DG increased 1.7 and 3.2 folds expression of brain $p47^{phox}$ and $gp91^{phox}$, respectively ($p<0.05$). AA intake down-regulated the expression of

p47^{phox} and gp91^{phox}, and dose-dependent manner was presented in lowering gp91^{phox} expression (p<0.05).

AA treatments reduced brain glycative and inflammatory stress

DG treatment increased 5.2 folds AGE, 4.9 folds CML and 4.3 folds RAGE expression (Figure 3, p<0.05). AA intake at 5, 10 and 20 mg/kg/day decreased 24.3-55.6% AGE and 28.5-54.3% CML expression, and at 10 and 20 mg/kg/day lowered 27.0-28.1% RAGE expression when compared with DG treatment alone (p<0.05). DG injection up-regulated MAPK expression (Figure 4, p<0.05). AA intake dose-dependently suppressed 14.2-46.5% p-p38 expression; and at 10 and 20 mg/kg/day down-regulated 28.2-43.6% p-JNK expression (p<0.05). DG injection raised brain IL-6 and TNF-alpha levels (Table 3, p<0.05). AA intake dose-dependently decreased brain IL-6 and TNF-alpha levels (p<0.05). As shown in Figure 5, DG enhanced 3.1 folds CD11b expression (p<0.05). AA intake at 10 and 20 mg/kg/day declined 27.3-28.6% CD11b expression when compared with DG treatment alone (p<0.05).

AA treatments declined GFAP expression

DG treatment down-regulated 70.4% BDNF expression and raised 2.9 folds GFAP expression (Figure 6, p<0.05). Compared with DG treatment alone, AA intake at 20 mg/kg/day restored 43.2% BDNF expression, and at 10 and 20 mg/kg/day decreased 37.5-39.4% GFAP expression (p<0.05).

Discussion

Human brain aging was highly associated with oxidative stress and neuron apoptosis (Cruz-Sánchez et al. 2010). These characteristics including morphological evidence of cell

death were reflected in brain tissues of DG treated mice (Wu et al. 2011; Han et al. 2014). Our present study found that AA intake at 10 and 20 mg/kg/day increased its deposit in brain of DG treated mice, which contributed to restrict protein expression of Bax, cleaved caspase-3, NAPDH oxidase, AGE, RAGE, CD11b and MAPK; and decrease ROS and inflammatory cytokines production. Furthermore, AA intake lowered GFAP expression in brain of DG treated mice. These findings indicated that this triterpene could penetrate blood brain barrier, and execute anti-apoptotic, anti-oxidative and anti-glycative protection for brain.

Bcl-2 is an anti-apoptotic factor, and Bax and caspase-3 are pro-apoptotic factors. We found AA intake at 10 and 20 mg/kg/day substantially down-regulated Bax and cleaved caspase-3 expression, and mildly retained Bcl-2 production, which in turn diminished apoptotic stress in brain of DG treated mice. These findings revealed that the anti-apoptotic effect of AA was mainly due to it decrease pro-apoptotic factors. The activation of NADPH oxidase is an important source of ROS in neurons, and responsible for oxidative cell death in neurodegenerative diseases (Sun et al. 2007). We found that AA treatments effectively down-regulated brain protein expression of $p47^{phox}$ and $gp91^{phox}$, cytosolic and membrane components of NADPH oxidase, respectively. These findings supported the anti-oxidative protection of AA against DG-induced oxidative stress, and also explained the lower ROS level in brain of AA treated mice. Dkhar and Sharma (2011) indicated that brain protein carbonyl could serve as an oxidative biomarker relevant to aging. In our present study, AA intake markedly decreased brain protein carbonyl level, as well as retained GSH content and GPX activity in brain of DG-treated mice. These data also agreed that this triterpene retarded oxidative progression in brain of DG-treated mice. On the other hand, it is reported that ROS stimulates Bax relocalization and caspase activation (Kirkland and Franklin 2003).

Since AA intake already reduced ROS formation in brain, the lower protein expression of Bax and cleaved caspase-3 could be explained. These results suggest that AA provided anti-apoptotic effects for brain partially via its anti-oxidative action.

Glycative stress from AGE overproduction is a risk factor responsible for brain aging (Lu et al. 2010). The presence of CML, a predominant AGE, could be detected in brain neurons of subjects with normal aging, and patients with Alzheimer's disease (Dei et al. 2002). RAGE expression in brain tissue increases with age, and is involved in aging related injury (Thangthaeng et al. 2008). In our present study, AA intake effectively decreased brain AGE, CML and RAGE expression in DG-treated mice. Obviously, this compound greatly diminished brain glycative stress in those mice. AGEs formation and oxidative stress are mutually enhanced, and both tightly linked to aging process (Peppa et al. 2008). Thus, the lower AGEs production in brain of AA-treated mice could be partially ascribed to the anti-oxidative activity of AA. Furthermore, the reduced AGEs and RAGE production contributed to the less interaction of AGEs and RAGE; and in turn declined MAPK activation, which was evidenced by the limited phosphorylation of p38 and JNK in brain of AA-treated mice. It is known that MAPK activation promotes the generation of inflammatory cytokines in neuronal cells (Dukic-Stefanovic et al. 2003). Thus, the down-regulation of MAPK consequently lowered IL-6 and TNF-alpha formation in brain of AA-treated mice. In addition, it is reported that microglial activation stimulates the release of inflammatory mediators including IL-6 and TNF-alpha, and is evidenced by increased brain CD11b expression (Ryu et al. 2009; Tatar et al. 2010). Our data revealed that AA intake at 10 and 20 mg/kg/day decreased CD11b expression, which in turn mitigated brain inflammatory response. These findings suggest that AA could alleviate DG-induced brain inflammation

through restricted MAPK and microglial activations.

Age-related hypertrophy of astrocytes, also called astrogliosis, was detected as an increase in GFAP (Nichols 1999), which caused the loss of synaptic functions and/or cognitive defects (Jin et al. 2014). In our present study, AA intake at 10 and 20 mg/kg/day reduced brain GFAP expression, which suggested that this compound might be able to retard astrogliosis. BDNF is responsible for synaptic integrity and synaptic plasticity (Dwivedi 2013), and its expression was decreased in the hippocampus and frontal cortex in patients with Alzheimer's disease (Siegel and Ghauhan 2000). We found that AA intake at high dose retained brain BDNF expression, which might benefit neurons survival and synaptic integrity for those DG-treated mice. The decreased GFAP and retained BDNF from AA treatments implied that this compound might improve aging related synaptic functions. It was interesting to find that AA intake at 20 mg/kg/day led to lower AA deposit in brain of DG treated mice than that of non-DG treated mice. It is highly possible that DG induced brain injury impaired AA deposit. This finding implied that using AA for brain protection should consider AA bioavailability in this tissue. AA is a triterpene naturally occurring in several plant foods. Our previous study reported that AA administration at 10 and 20 mg/kg/day alleviated high fat diet induced hepatotoxicity in mice, and did not induce any sign of toxicity (Yan et al. 2014). Thus, this agent at these doses seems safe for application.

In conclusion, the intake of asiatic acid increased its deposit in brain of senescent mice. Asiatic acid treatments at 10 and 20 mg/kg/day protected brain against apoptotic, oxidative and glycative stress via decreasing ROS and AGE levels; down-regulated Bax, NADPH oxidase, RAGE and MAPK expression. This triterpene also declined brain GFAP expression. Therefore, the supplement of asiatic acid or foods rich in this compound might be helpful for

the prevention or alleviation of aging.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

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Table 1

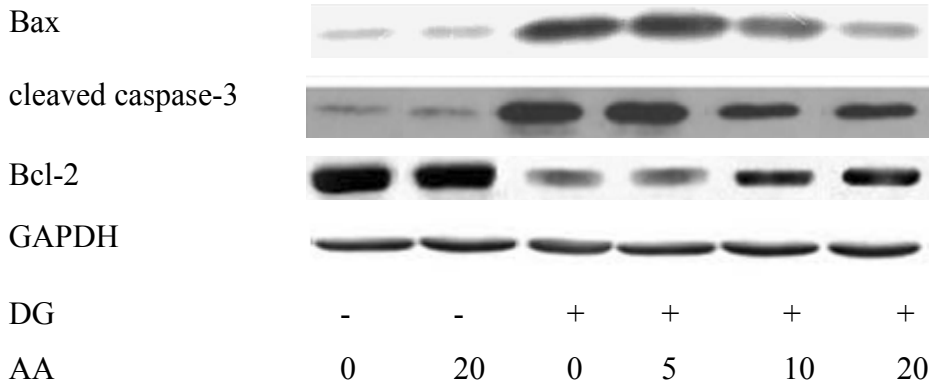
Body weight, water intake, feed intake, brain weight and brain AA content in mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Values are mean ± SD, n = 10.

	Body weight g/mouse	Water intake ml/mouse/day	Feed intake g/mouse/day	Brain weight g/mouse	AA content nmol/mg protein
AA-0	30.3±1.1[a]	2.1±0.4[a]	2.3±0.6[a]	0.42±0.07[a]	-*,[a]
AA-20	29.7±0.8[a]	2.3±0.7[a]	2.0±0.4[a]	0.51±0.05[a]	0.29±0.05[d]
DG+AA-0	31.0±1.4[a]	2.2±0.5[a]	2.2±0.7[a]	0.45±0.06[a]	-[a]
DG+AA-5	30.7±1.2[a]	2.5±0.6[a]	2.1±0.3[a]	0.52±0.04[a]	-[a]
DG+AA-10	31.4±1.0[a]	2.3±0.4[a]	2.4±0.6[a]	0.47±0.05[a]	0.08±0.04[b]
DG+AA-20	29.9±1.3[a]	2.0±0.6[a]	2.0±0.5[a]	0.48±0.06[a]	0.19±0.09[c]

*Means too low to be detected.

[a-d]Means in a column without a common letter differ, $p<0.05$.

Fig. 1. Protein expression of Bax, cleaved caspase-3 and Bcl-2 in brain from mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). [a-d]Means among bars without a common letter differ, $p<0.05$.



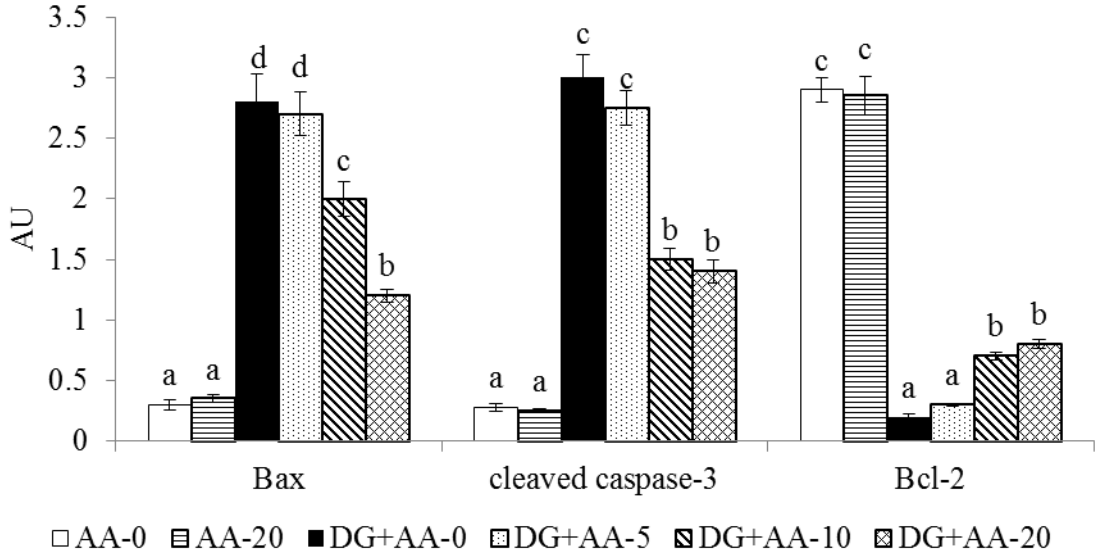


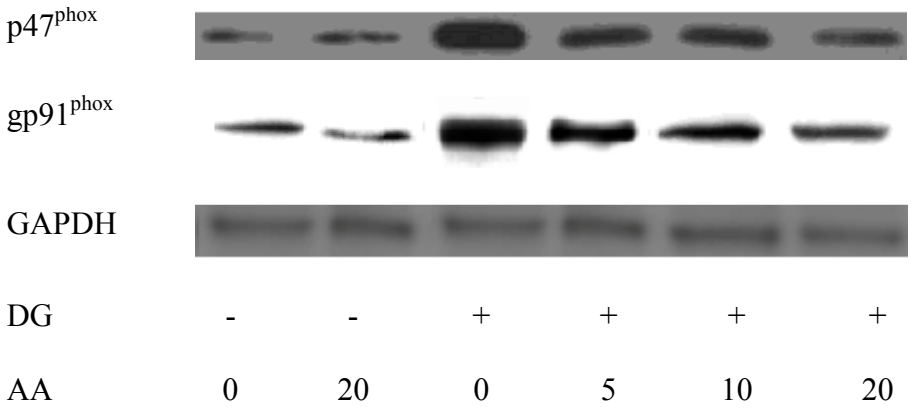
Table 2

ROS, protein carbonyl and GSH levels, and GPX activity in brain from mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Values are mean ± SD, n = 10.

	ROS RFU/mg protein	Protein carbonyl pmol/mg protein	GSH ng/mg protein	GPX U/mg protein
AA-0	0.18±0.03[a]	12.1±0.6[a]	89±2[e]	21.9±0.4[e]
AA-20	0.17±0.05[a]	10.7±0.5[a]	98±3[f]	22.8±0.5[e]
DG+AA-0	1.98±0.19[e]	147.7±7.1[e]	45±2[a]	10.1±0.3[a]
DG+AA-5	1.52±0.15[d]	120.4±6.0[d]	53±4[b]	12.3±0.6[b]
DG+AA-10	1.08±0.10[c]	98.5±2.8[c]	63±6[c]	15.5±0.4c
DG+AA-20	0.73±0.11[b]	65.8±3.1[b]	76±5[d]	18.6±0.5[d]

[a-f]Means in a column without a common letter differ, $p<0.05$.

Fig. 2. Protein expression of brain p47^{phox} and gp91^{phox} in mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). $^{a-e}$Means among bars without a common letter differ, $p<0.05$.



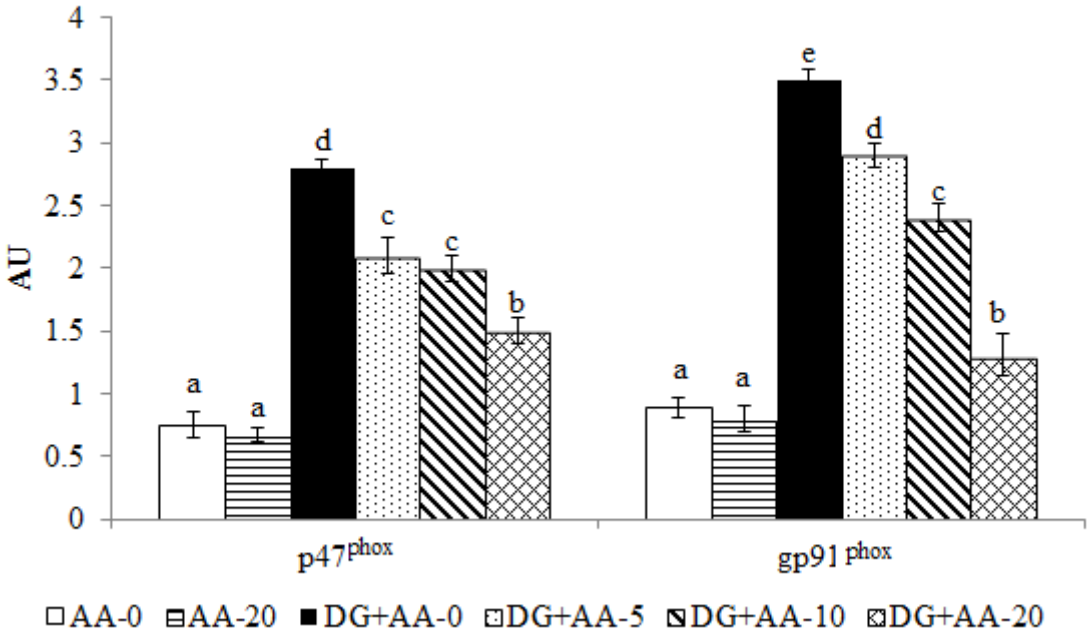
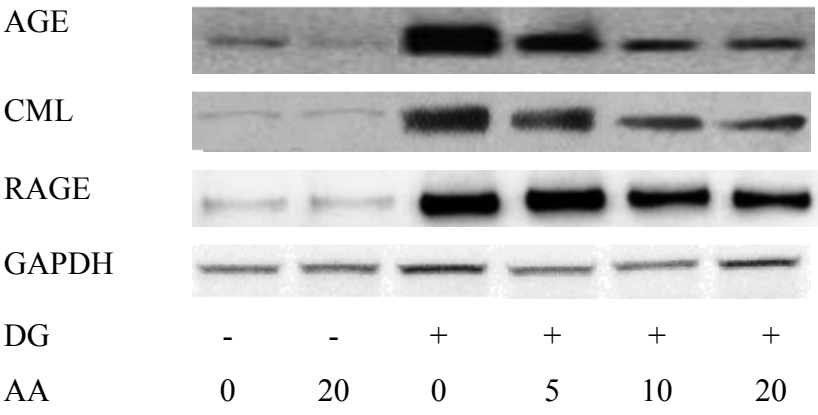


Fig. 3. Protein expression of brain AGE, CML and RAGE in mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). [a-d]Means among bars without a common letter differ, $p<0.05$.



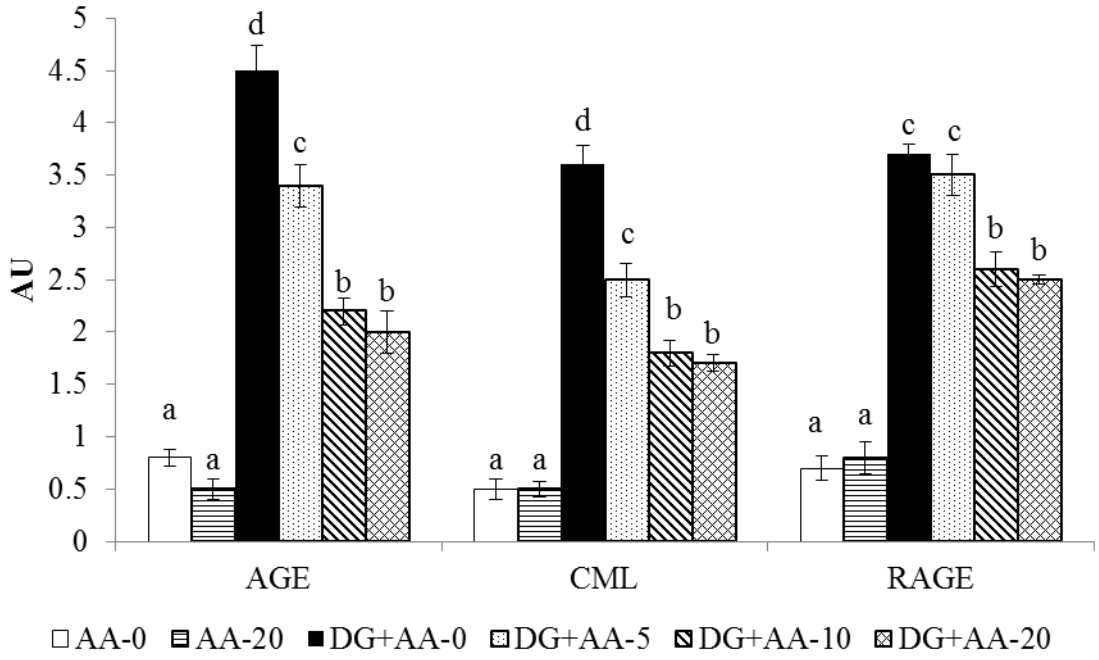
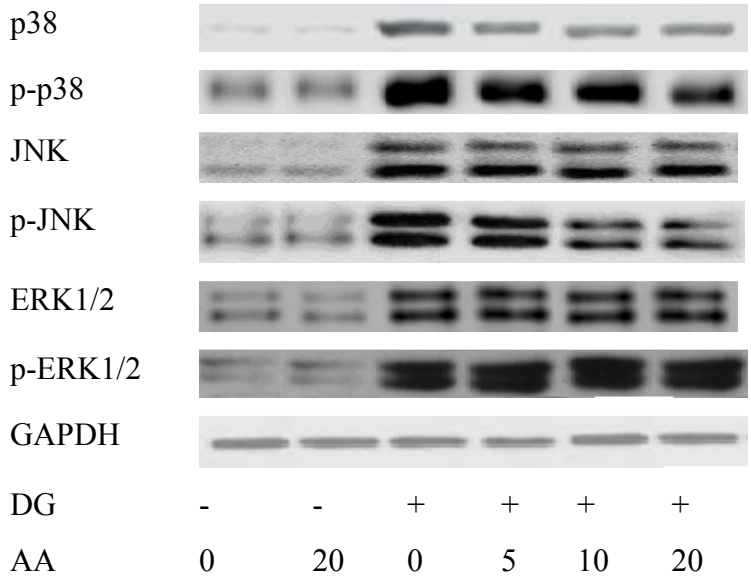


Fig. 4. Protein expression of MAPK in brain from mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). [a-e]Means among bars without a common letter differ, $p<0.05$.



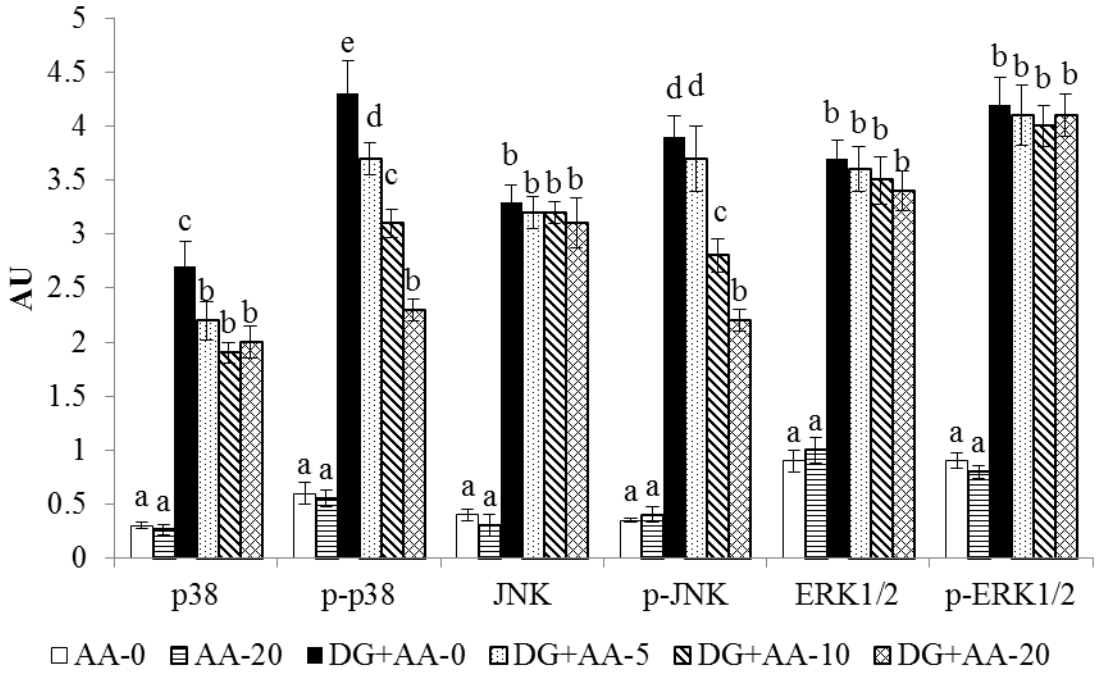


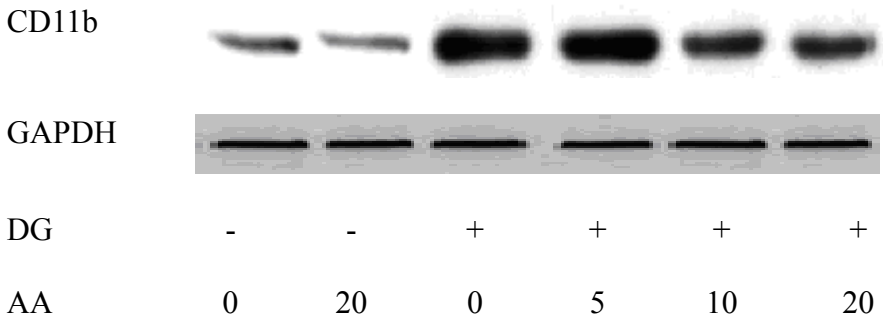
Table 3

IL-6 and TNF-alpha levels in brain from mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Values are mean ± SD, n=10.

	IL-6 pg/mg protein	TNF-alpha pg/mg protein
AA-0	1.06±0.14[a]	1.18±0.21[a]
AA-20	0.99±0.09[a]	1.10±0.17[a]
DG+AA-0	3.34±0.31[e]	4.88±0.45[e]
DG+AA-5	2.90±0.16[d]	4.25±0.36[d]
DG+AA-10	2.34±0.23[c]	3.41±0.22[c]
DG+AA-20	1.72±0.14[b]	2.20±0.27[b]

[a-e]Means in a column without a common letter differ, $p<0.05$.

Fig. 5. Protein expression of brain CD11b in mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). [a-c]Means among bars without a common letter differ, $p<0.05$.



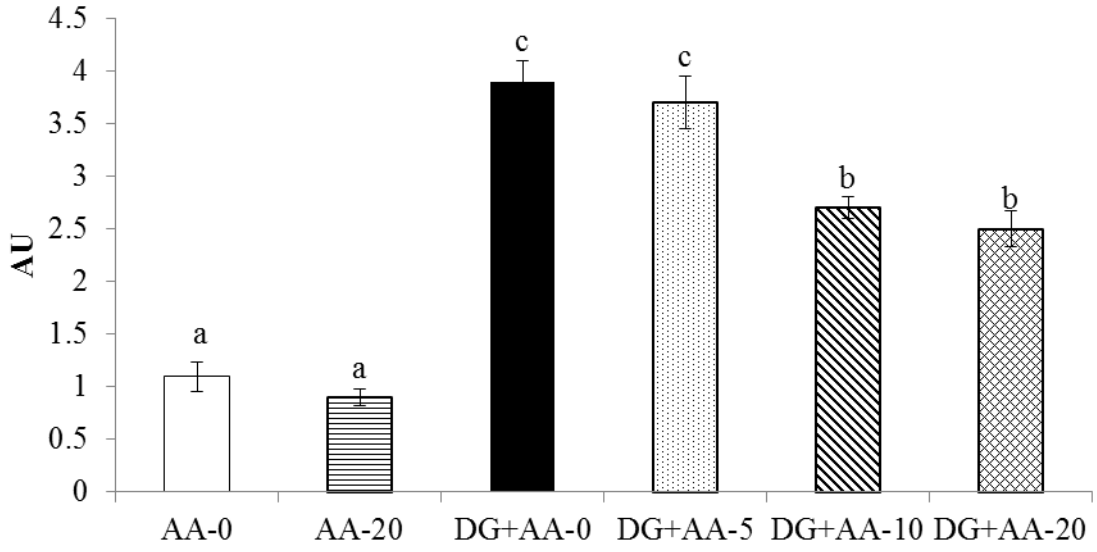
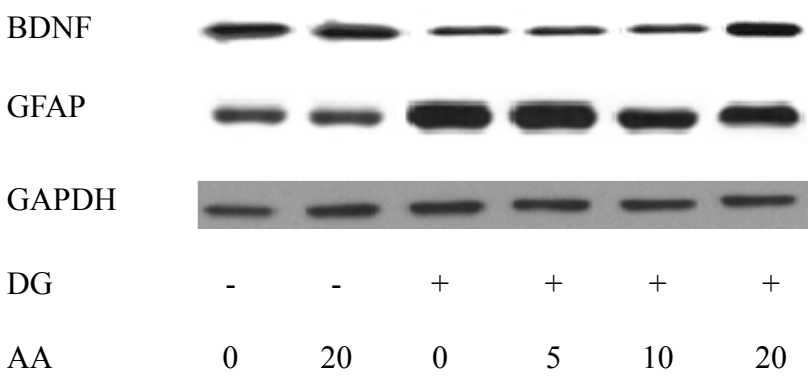
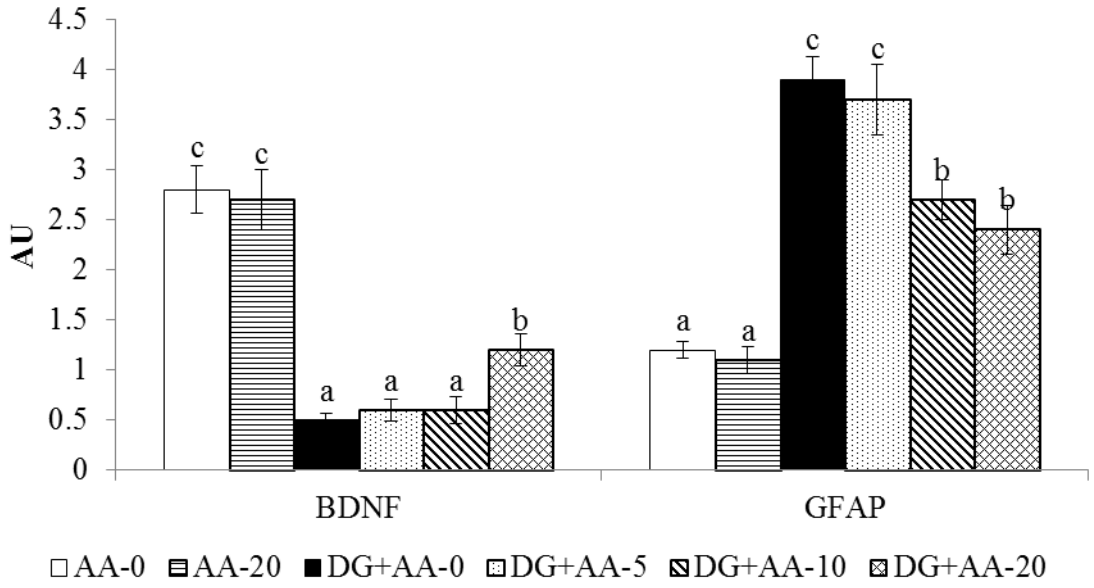


Fig. 6. Protein expression of brain BDNF and GFAP in mice with or without DG treatment and consumed AA at 0, 5, 10 or 20 mg/kg/day for 8 weeks. Data are mean ± SD (n = 10). [a-c]Means among bars without a common letter differ, $p<0.05$.





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