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Daidzein Augments Cholesterol Homeostasis via ApoE to Promote Functional Recovery in Chronic Stroke

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Stroke is the world’s leading cause of physiological disability, but there are currently no available agents that can be delivered early after stroke to enhance recovery. Daidzein, a soy isoflavone, is a clinically approved agent that has a neuroprotective effect in vitro, and it promotes axon growth in an animal model of optic nerve crush. The current study investigates the efficacy of daidzein on neuroprotection and functional recovery in a clinically relevant mouse model of stroke recovery. In light of the fact that cholesterol is essential lipid substrates in injury-induced synaptic remodeling, we found that daidzein enhanced the cholesterol homeostasis genetic program, including Lxr and downstream transporters, Apoe, Abca1, and Abcg1 genes in vitro. Daidzein also elevated the cholesterol homeostasis genes in the poststroke brain with Apoe, the highest expressing transporter, but did not affect infarct volume or hemispheric swelling. Despite the absence of neuroprotection, daidzein improved motor/gait function in chronic stroke and elevated synaptophysin expression. However, the daidzein-enhanced functional benefits and synaptophysin expression were abolished in Apoe-knock-out mice, suggesting the importance of daidzein-induced ApoE upregulation in fostering stroke recovery. Dissociation between daidzein-induced functional benefits and the absence of neuroprotection further suggest the presence of nonoverlapping mechanisms underlying recovery processes versus acute pathology. With its known safety in humans, early and chronic use of daidzein aimed at augmenting ApoE may serve as a novel, translatable strategy to promote functional recovery in stroke patients without adverse acute effect.

Key words: ApoE; cholesterol transporter; daidzein; motor/gait function; stroke recovery

Significance Statement

There have been recurring translational failures in treatment strategies for stroke. One underlying issue is the disparity in outcome analysis between animal and clinical studies. The former mainly depends on acute infarct size, whereas long-term functional recovery is an important outcome in patients. In an attempt to identify agents that promote functional recovery, we discovered that an FDA-approved soy isoflavone, daidzein, improved stroke-induced behavioral deficits via enhancing cholesterol homeostasis in chronic stroke, and this occurs without causing adverse effects in the acute phase. With its known safety in humans, the study suggests that the early and chronic use of daidzein serves as a potential strategy to promote functional recovery in stroke patients.

Introduction

Improvements in acute stroke patient care have resulted in reduced stroke mortality, leaving more survivors with severe disability and a long road of recovery. Despite demands in developing potential strategies to aid functional recovery in stroke, there is a paucity of medical treatments available to treat post-stroke impairment and promote recovery. Studies showed that stroke initiates metabolic and genetic changes that persist for weeks to months following the initial insult (Carmichael, 2006;
Nudo, 2007). However, understanding of the mechanism underlying the repair/restorative processes and identification of biological targets in chronic stroke is largely limited.

Consumption of soy is associated with numerous health benefits against obesity, cancer, osteoporosis, cardiovascular disease, and immune deficiency (Orgaard and Jensen, 2008; Wenzel et al., 2008). Daidzein, an isoflavone, is a major component of soy with structural similarity to estrogen. It exerts an anti-inflammatory effect, lowers lipid levels, and increases mitochondrial biogenesis (Ricketts et al., 2005; Rasbach and Schnellmann, 2008; Wang et al., 2008). As an activator of nuclear receptor peroxisome proliferator-activated receptors (PPARs), daidzein enhances transcription of PPAR-dependent genes, including liver X receptors (LXRs, Nrhl gene family in mice). By heterodimerizing with retinoid X receptors (RXRs), LXRs regulate the transcription of cholesterol transporter genes, Apoe, Abca1, and Abcg1 (Whitney et al., 2002), either directly or through sterol-independent regulatory element and enhancer sites (Shih et al., 2000; Kennedy et al., 2001; Langmann et al., 2002).

The cholesterol transporters provide lipid substrates for maintenance of membrane and synaptic integrity in normal and injury-induced synaptic remodeling. Reduced availability of lipid substrates is associated with neurodegenerative conditions, injury-induced synaptic remodeling. Reduced availability of lipid in vivo ing naive ApoE, whereas Abcg1 acts on partially lipidated ApoE program with Apoe being a critical component.

In another study, Wang et al. (2008) reported that daidzein, without affecting infarct size, promoted underlying events that are associated with these benefits. Here we report that daidzein is a potential neuroprotective and recovery agent in stroke and plasticity, and it promotes axonal outgrowth in cultured hippocampal neurons via estrogen receptor signaling (Wang et al., 2008). Previously, we reported that daidzein overcame the inhibition of axonal outgrowth induced by myelin-associated glycoproteins in vitro and promoted regeneration of axons in an optic nerve crush model in vivo (Ma et al., 2010). Furthermore, studies by others reported that daidzein exerted neuroprotection in oxygen-glucose deprived conditions (Hurtado et al., 2012) and enhanced recovery in rats following stroke (Hurtado et al., 2012; Stout et al., 2013). Because daidzein is a fairly safe agent that has been widely consumed as a soy component, we investigated whether daidzein is a potential neuroprotective and recovery agent in stroke and underlying events that are associated with these benefits. Here we report that daidzein, without affecting infarct size, promoted functional recovery via enhancing the cholesterol homeostasis program with Apoe being a critical component.

Materials and Methods

Study design. Experiments were performed with in vitro culture systems and in vivo using mice. For in vitro studies, three independent experiments were performed with duplicated samples at a given drug concentration. For in vivo studies, the use of animals and procedures was approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University and in accordance with the Institutional Animal Care and Use Committee, National Institutes of Health, and ARRIVE guidelines. The number of animals was calculated a priori by power calculation. Eleven animals per group were targeted to reach power 0.83 at a significance level of <0.05 assuming 25% difference in mean, a 20% SD at the 95% confidence level. Mice were randomized to receive sham or middle cerebral artery occlusion (MCAO) surgery. In certain cases, surgeries could not be blinded to the identity of groups at the time of surgery due to higher body weight or phenotype associated with Apoe knock-out (KO) mice. Mice were randomly selected by drawing different colored balls to receive vehicle or daidzein. The identity of the drug was concealed (coded A or B) by a third party and administered to animals blinded to experimenters. Because of the chronic nature of the study, each animal received a tattoo and their identity and treatment were blinded to the persons who assessed injury size and performed the behavior tests.

Cell cultures. HT22 murine hippocampal cells were cultured in DMEM with 4500 mg/dl glucose (Sigma-Aldrich), 1-glutamate, and pyridoxine hydrochloride. C8-D1A immortalized mouse astrocyte cells (CRL-2541, American Type Culture Collection) were cultured in the DMEM. These cultures were supplemented with 10% FBS (Mediatech), 100 IU penicillin and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified 5% CO2 incubator.

Primary neuron-enriched cultures were generated from the papain digestion of cortical tissue harvested from E14.5 embryos of C57 mice. The resulting cells were plated at a density of 1.04 × 105 cells/cm2 in 6-well plates in Neurobasal media supplemented with 2% B27, 0.5 mM glutamine, and penicillin/streptomycin. After 1 d in culture, the cells were maintained in media containing 10 μM 5′-fluoro-2′-deoxyuridine to kill off non-neuronal cells. The cells were treated after 7 DIV with media lacking mitotic inhibitors and consisted of >99% neurons as assessed by MAP2 and GFAP immunocytochemistry (data not shown).

For astrocyte cultures, cortical tissue was harvested from postnatal day 1 mice, digested with papain, and plated at 1.5 × 105 cells/cm2 in 6-well plates in MEM (Mediatech) supplemented with 10% horse serum and penicillin/streptomycin. After the astrocytes reached confluency (~2 weeks), the cultures were treated with 8 μM cytosine-D-arabinofuranose for 3 d to kill off contaminating nonastrocyte cells (Haskell-Layton et al., 2010).

Daidzein and T0901317 treatment in vitro culture. Neurons and astrocytes at 7 and 14 DIV, respectively, were treated with daidzein (Sigma-Aldrich) and T0901317 (Tocris Bioscience). The indicated concentrations of daidzein and T0901317 were added in the culture media with the absence of mitotic inhibitors. Twenty-four hours later, mRNA was harvested using TRI Reagent (Molecular Research Center) and purified using the Direct-zol RNA Miniprep kit (Zymo Research) with in-column DNasel digestion.

ApoE promoter activity assay. U373 human glioblastoma cells were maintained in MEM, supplemented with 10% FBS (Atlanta Biologicals) and an antibiotic-antimycotic mixture (Mediatech). The day before transfection, cells were transferred to 96-well plates at a density of 50,000 cells per well. Cells were transfected with either the pGL3-Basic vector (Promega) or the pGL3 vector containing 1.1 kb of the 5′ upstream regulatory promoter region of the human ApoE gene controlling expression of the firefly luciferase reporter gene (hApoE1). Cells were simultaneously transfected with 285 ng of the pGL3-based plasmid (vector or hApoE1) and 15 ng of the pRLSV40 plasmid (Promega) expressing renilla luciferase under the constitutive SV40 promoter as an internal control. Plasmid DNA was transferred into the cells using 0.75 μl per well of the Transfectin transfection reagent (Bio-Rad) in serum- and antibiotic-free medium. Two hours after transfection, cells were treated with vehicle or the indicated concentrations of daidzein in MEM containing 10% FBS and antibiotics for 48 h with n = 6 per treatment. Cells were then lysed, and both firefly and renilla luciferase activities were measured using the Dual Luciferase kit (Promega), per the manufacturer’s instructions. Results are expressed as the ratio of firefly/renilla luciferase activity. Toxicity was evaluated by a lactate dehydrogenase enzyme activity assay kit, per the manufacturer’s instructions (Sigma-Aldrich).

Animals. Experiments were performed in 10- to 11-week-old male C57 (C57 bl/6) and Apoe KO (C57 background) mice purchased from The Jackson Laboratory. The mice were housed at the institute’s animal facility, which maintained temperature, humidity, and 12 h light/dark cycle. A maximum of 5 mice was housed in a cage with an individual ventilating system and irradiated bedding (1/8″ Bed O’s Cobs, Anderson). Sterilized food (PicoLab Rodent diet 5053, LabDiet) and water were freely accessible in their cage.

Acute and stroke recovery models and daidzein treatment. Transient MCAO was performed in mice according to previously described methods (E. Kim et al., 2012; Qin et al., 2014). Mice were anesthetized with isoflurane (5% induction and 1.5%-2.0% maintenance) with a mixture
of oxygen and nitrogen (30%)/70%). A 0.6 Teflon-coated black monofilament surgical suture (Doccoll) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA and transiently occlude for 30 min. Cerebral blood flow was continuously monitored 15 min before stroke, during 30 min MCAO and 15 min of reperfusion by Laser-Doppler flowmetry (Periflux System 5010; Perimed). Mice were then placed in a recovery cage until the animal regained consciousness and reactivity, using a rectal probe controlled by Masterflex water pump on an operating table and thermistor temperature controller (Cole-Parmer). Animal’s body temperatures were maintained at 37 ± 0.5°C during entire surgical procedures and recovery after the surgery. The mice were then returned to their home cages where they were previously housed together. Buprenorphine, lidocaine, and bupivacaine were administered during postischemia as analgesics. Inclusion and exclusion criteria for mice were based on the severity of ischemia. Animals exhibiting reduced cerebral blood flow >80% during MCAO and restored cerebral blood flow >80% by 10 min following reperfusion were included in the study.

Our proximal MCAO by an intraluminal thread method produced an infarct ~40 mm³ (~30% of a hemisphere) and mostly confined to the striatum with incidental damage in somatosensory cortex. The size of the basal ganglia in the rodent brain as a proportion of total brain volume is approximately twofold higher than that in the human brain (Swanson, 1995). Therefore, subcortical infarcts produced by these methods would be approximately twofold larger than those in humans when anatomical proportions between the species are considered. As subcortical stroke in humans ranges from 4.5% to 14% of a hemisphere (Carmichael, 2005), the current mouse stroke model generates proportionally similar magnitudes of subcortical injury as human subcortical stroke.

For long-term stroke recovery, mice received moxifloxacin (100 mg/kg) for 3 d. The prophylactic antibiotic treatment was shown to effectively reduce mortality in an animal model of stroke by attenuating peripheral infection (Meisel et al., 2004). In addition, saline was subcutaneously administered daily, and hydrogel (Clear H2O) was given to prevent dehydration. With the implementation of poststroke care (antibiotic regimen, rehydration, and feeding hydrogels with soft diet) during the acute period (<1 week), mice start to regain their body weight by day 5 and continue to recover from stroke. Animals were randomly selected for vehicle or daidzein treatment. Vehicle or daidzein (10 mg/kg, Sigma-Aldrich) was administered subcutaneously within 30 min of reperfusion after confirming the reperfusion of blood flow, daily for 7 d and then every other day up to 1 month.

**Tissue preparation.** Brains were excised, frozen, and sectioned using an unbiased stereological sampling strategy to reflect the MCA territory in both hemispheres as previously described (E. Kim et al., 2014). Tissue sections (30 μm thickness) were collected serially at 600 μm intervals using a cryostat (thickness, 40 μm) and incubated overnight with the following primary antibodies: GFAP (ab8049, Abcam), PSD-95 (51-6900, Invitrogen), or GAPDH (sc-25778, Santa Cruz Biotechnology) antibody in blocking buffer (1:1000) at 4°C. The membrane was washed with Tris-buffered saline containing 0.05% Tween 20 followed by incubating with appropriate secondary antibodies conjugated with AlexaFluor-680 (A 21088, Invitrogen), IRDye 800CW (926-32212, Li-Cor), or IRDye 680RD (926-68071, Li-Cor) in blocking buffer for 1 h. Then each protein’s specific band was visualized using the Odyssey Imaging System (Li-Cor). Specificity of bands was confirmed in tissues from Apo KO mice (for ApoE band) and/or by omitting primary antibody incubation. Western blots were performed in multiple gels. To normalize inter-blot variability, identical samples were loaded in each blot as internal controls, and the density of the internal standard sample (normalized by GAPDH or β-actin) was used to standardize other samples in multiple blots.

**Immunohistochemistry.** Mice (n = 2 or 3/group) were perfusion-fixed, and immunohistochemistry was performed for visualization of protein localization, according to the method previously described (Qin et al., 2014). Brains were sectioned coronally in a cryostat (thickness, 40 μm) and incubated overnight with the following primary antibodies: GFAP (astrocytes marker, 1:3000; EMD Millipore), ApoE (1:900; Abcam), MAP-2 (neuronal marker, 1:3000, Abcam), followed by incubation with appropriate secondary antibodies conjugated with AlexaFluor-488 or -594 (1:200; Invitrogen) for 1 h at room temperature. Specificity of the staining was confirmed in the brain section from Apo KO mice (for ApoE) and omitting primary antibody incubation. Sections were washed with PBS between incubations and examined under a florescent microscope or laser scanning confocal microscopy (Carl Zeiss).

**Behavior tests.** Motor and gait functions were longitudinally assessed by a rotarod (Med Associates) and Noldus Catwalk XT gait analysis system (Noldus Information Technology), as previously reported (Qin et al., 2014). For rotarod test, mice were placed on a rod of rotarod device, which was set to accelerate from 4 to 40 rpm over the course of 5 min. After 1 week of daily pretraining, the latency to fall from the rod was averaged from five trials. For Catwalk, mice were pretrained daily for 2 weeks to cross an illuminated glass walkway 3 consecutive times and then at intervals during the poststroke period. The images from each trial that consisted of 3 consecutive runs were converted into digital signals. Each footprint was classified as a left front (LF), left hind (LH), right front (RF), and right hindpaw (RH). After the classification, a priori selected gait parameters that are relevant to the kinematics of stroke recovery were analyzed: spatial parameters based on individual paws (mean intensity), relative position between paws (stride length), temporal parameters (swing speed, walk speed), and parameter related to interlimb coordination (regularity index).

**Data analyses.** Infarct volume and %HS were reported as mean ± SEM. Gene expression levels from in vivo studies were presented as the β-actin normalized value according to the formula, Value = (2^β-actin threshold cycle-target gene’s threshold cycle). Gene expression levels in vitro studies were
reported relative to control cultures and averaged from three independent experiments.

Statistics. Comparison between two groups was statistically evaluated using Student’s t test. Multiple comparisons were made using ANOVA followed by post hoc tests. For gene/protein expression, analyses in cultures and the brain were performed by one-way and two-way ANOVA, respectively, followed by post hoc Newman–Keuls tests. For gait analyses, two-way ANOVA with Bonferroni correction was used. Differences were considered significant at p < 0.05.

Results
Daidzein increases Lxr, Apoe, Abca1, and Abcg1 gene expression in vitro
The effect of daidzein on the transcription of genes involved in cholesterol homeostasis, including Lxr and downstream transporters Apoe, Abca1, and Abcg1, was assessed in primary cortical neurons and astrocytes. Daidzein increased expression of Lxr, a PPARγ-dependent gene, more robustly in neurons than astrocytes (Fig. 1A, B). In astrocytes, daidzein treatment significantly elevated Abca1 and Apoe (Fig. 1D, H), but not Abcg1 mRNA (Fig. 1F). Treating the cultures with T0901317, an LXR agonist, showed expected increases in expression of Lxr downstream transporter genes (Fig. 1C–H) but not its own expression (Fig. 1A, B). T0901317 also increased sterol-regulatory element binding proteins-1 (Srebp1) and its target genes, fatty acid synthase (Fas) and lipoprotein lipase (Lpl) in astrocytes (Fig. 1J, L, N). Daidzein increased Srebp1 in neurons (Fig. 1I), without affecting Fas and Lpl expression both in neurons and astrocytes (Fig. 1K–N). We further determined transcriptional activity of Apoe in human glioblastoma U373 cells containing a 1.1 kb of human Apoe (hAPOE) promoter-luciferase plasmid (Maloney et al., 2007). Incubation with different concentrations of daidzein, from 5 to 100 μM, increased Apoe transcriptional activity (Fig. 1O). Lactate dehydrogenase levels in the media in cultures treated with various concentrations of daidzein were not different from vehicle-treated cultures, excluding potential toxicity of daidzein at the concentrations used (Fig. 1P). The findings show that daidzein induces the cholesterol homeostasis genetic program without toxicity and inducing transcription of the lipogenic genes.

Stroke induces Lxr-downstream transporter gene expression
To address the relevance of the cholesterol homeostasis genetic program in chronic stroke, we first determined temporal changes in Lxr and transporter Abca1, Abcg1, and Apoe genes in the acute and recovery phase of poststroke brain (Fig. 2A–D). While relatively constant in the contralateral hemisphere, these genes were induced in the stroke hemisphere.
during 1–4 weeks after stroke, followed by a return to baseline at ~2 months after stroke. Of these genes, Apoe was the most abundantly expressed cholesterol transporter in the brain. The basal level of Apoe transcripts (Apoe/Actin; 0.84 ± 0.06, n = 4–6) was ~50–100 times higher than those of Abca1 (0.0064 ± 0.0002) and Adeg1 (0.013 ± 0.007). Apoe protein levels were noticeably elevated at 1 month after stroke but not at day 3, reflecting corresponding protein expression at this time (Fig. 2E). ApoE protein was predominantly localized in astrocytes in the contralateral (Fig. 2H) and rarely in the peri-infarct area of stroke hemisphere (Fig. 2I,N). On the other hand, ApoE staining was mostly absent in neurons in the contralateral hemisphere (Fig. 2L) with some occurrence in the peri-infarct area (Fig. 2M,N), supporting a reported view on -axis represents mRNA normalized by actin. *p < 0.05 versus contralateral (Student’s t test). **p < 0.01 versus contralateral (Student’s t test). ***p < 0.001 versus contralateral (Student’s t test). E, ApoE protein levels in the brain of Pre, 3 d, and 1 month after stroke (cropped images of original blots). ApoE−/−, brain tissue from ApoeKO mice, *p < 0.05 (two-way ANOVA, followed by a Newman–Keuls post hoc test). ***p < 0.001 (two-way ANOVA, followed by a Newman–Keuls post hoc test). F–M, Immunolocalization of ApoE in 1 month poststroke brain. Double immunofluorescence labeling of ApoE (F) with GFAP (G–I) or ApoE (J) with MAP2 (K–M). Merged micrographs in the contralateral cortex (H, I), peri-infarct area in the ipsilateral hemisphere (J, M), F, Inset. No specific ApoE immunoreactivity in the brains from ApoeKO mice. N, GFAP-stained ipsilateral hemisphere. Square represents the peri-infarct area chosen for I, M, n = 2. Scale bars: I, 100 μm; N, 1 mm.

Daidzein increases the stroke-induced cholesterol homeostasis program without reducing infarct size

We initially assessed the effect of daidzein on multiple genes that are involved in cholesterol homeostasis and synaptic remodeling at day 3 after stroke. Ratios of stroke-induced mRNA levels over those in the contralateral hemisphere (ipsilateral/contralateral) in vehicle-treated group showed increased Lxr, Scarb1, Abca1, and Gfap mRNA and the gene induction patterns were similar in daidzein-treated group (Fig. 3A; Table 1). For cholesterol homeostasis genes, we found that stroke significantly increased Lxr and Abca1 mRNA (Fig. 3B,C) and decreased in Adeg1 and Apoe genes (Fig. 3D,E). Among these genes, daidzein significantly increased Lxr and Apoe in the ipsilateral hemisphere (Fig. 3B,E). Both treatment groups displayed a similar degree of stroke severity indicated by comparable cerebral blood flow reduction during ischemia (14.6 ± 1.2 vs 14.1 ± 0.9, n = 25, not significant) and reperfusion at 10 min after stroke (97.2 ± 6.4% vs 118.2 ± 7.3%, not significant). Daidzein treatment neither increased nor decreased infarct size or edema (Fig. 3F), showing that daidzein has no acute neuroprotective effect.

A possibility of daidzein-induced long-term neuroprotection was assessed in mice treated with daidzein for 1 month. Ratios of stroke-induced mRNA levels against the contralateral hemisphere showed similar increases in Lxr, Scarb1, Abca1, Apoe, Tsp2, and Gfap mRNA in both vehicle and daidzein-treated groups (Fig. 4A; Table 2). Compared with vehicle treatment, daidzein significantly elevated Abca1, Adeg1, and Apoe mRNAs at 1 month after stroke (Fig. 4B–E). For lipogenic genes, daidzein increased stroke-induced Srebp1 mRNA without affecting Fas and Lpl expression (Table 2). ApoE protein was also significantly increased at this time (Fig. 4F). There was no difference in expression of the cholesterol homeostasis genes in age-matched sham mice treated with vehicle or daidzein for 1 month (data not shown). Because
stroke causes atrophy, brain volume representing noninjured tissue, ischemic scar tissue, remaining total ipsilateral tissue, and resorbed tissue (estimated infarct) were analyzed. We found no differences in any of these region volumes (Fig. 4G), confirming that daidzein-induced cholesterol homeostasis genetic program uncouples with neuroprotection.

Daidzein improves motor/gait function following stroke

As cholesterol homeostasis is a critical event for injury-induced repair and neural remodeling (Nudo, 2007), we next determined the effect of daidzein on functional recovery. To obtain sensitive and quantifiable functional data, behavior parameters that are biologically meaningful and relevant to the kinematics of stroke patients were chosen a priori for motor/gait function during acute and recovery phases following stroke. Stroke-induced changes in behavior were expressed as a percentage of the pre-ischemia baseline to account for interanimal variability. Stroke caused acute body weight reduction, which returned to baseline ~1 month in both groups. The acute body weight loss was less in daidzein-treated mice (Fig. 5A). Stroke caused acute and sustained deficits in locomotion in rotarod performance (Fig. 5B) and walking speed (Fig. 5C), which were improved by daidzein.
(indicated by #). Daidzein did not show benefit on the regularity index, a measure of interlimb coordination that spontaneously recovered by 2 weeks (Fig. 5D).

Several gait parameters for individual limbs were assessed in LF, LH, RF, and RH limbs. Following stroke, all four limbs showed sustained impairment in stride length (distance between paw prints). Chronic daidzein administration significantly reduced the deficit in LF, LH, and RH limbs (Fig. 6A). While stroke also caused sustained reduction in swing speed in all limbs in both groups, daidzein attenuated the deficit in RH limb (Fig. 6B).

Similar to the regularity index shown in Figure 5D, daidzein did not improve mean intensity, another spontaneously recovered parameter (Fig. 6C). Overall, the results showed that daidzein treatment improves motor/gait functions that showed sustained deficit, but did not gait parameters that were fully recovered, highlighting the efficacy of daidzein in a clinically meaningful context.

The functional benefits of daidzein treatment in the absence of neoprotection indicate that mechanisms underlying neuroprotection unlikely overlap with recovery/repair processes. In age-matched sham animals, chronic daidzein treatment did not affect the longitudinal gait functions compared with those of vehicle-treated group (data not shown).

**ApoE deficiency abolishes daidzein-enhanced motor/gait function in stroke**

As ApoE is the most abundant cholesterol transporter that was elevated by daidzein in vivo, we addressed the importance of daidzein-induced ApoE upregulation in functional recovery in A IPOe-knock-out (A IPOe KO) mice. Stroke resulted in acute body weight losses with subacute normalization by 2 weeks in vehicle-treated A IPOe KO mice. Chronic daidzein treatment did not attenuate acute body weight loss or improve rotarod performance (Fig. 7A, B). Daidzein also provided no functional benefits in gait functions (Fig. 7C–F). The only treatment effect (indicated by #) observed in A IPOe KO mice was the mean intensity in right RH, where daidzein treatment resulted in a greater deficit (Fig. 7G). Estimation of infarct volume by subtracting the ipsilateral from contralateral volumes at 1 month after ischemia showed no difference between the groups (Veh vs Dz, 20.2 ± 3.8 vs 25.8 ± 4.0 mm³, not significant, n = 13–14/group), confirming no detectable effect of daidzein on modulating infarct size. Treating A IPOe KO mice with daidzein increased Lxr and Abca1 gene expression at 1 month after stroke, showing that the absence of ApoE does not interfere with other cholesterol homeostasis genetic programs (Fig. 7H–J). Therefore, the findings suggest that daidzein-induced ApoE upregulation is a critical component in fostering functional recovery in chronic stroke.

**ApoE is necessary for daidzein-induced synaptophysin expression in chronic stroke**

We then determined whether the daidzein-induced ApoE upregulation is necessary for induction of synaptic elements after stroke. Chronic daidzein treatment in C57 mice selectively increased synaptophysin mRNA without altering Psd-95 at 1 month after stroke (Fig. 8A, B). Further analyses between the presynaptic and postsynaptic elements in C57 mice showed significant correlations between them in both hemispheres regardless of treatments (Fig. 8E, F). Notably, daidzein resulted in higher expression of synaptophysin at a given Psd-95 level in the ipsilateral hemisphere with a significant slope difference between the groups (p < 0.001; Fig. 8F), showing selective elevation of the presynaptic gene. Accordingly, there was a selective increase in stroke-induced synaptophysin protein in the daidzein-treated group (Fig. 8I). Compared with C57 mice, A IPOe KO mice showed an overall reduction of synaptophysin mRNA expression in both hemispheres (C57 vs A IPOe KO, Contralateral, 0.19 ± 0.02 vs 0.07 ± 0.04; Ipsilateral, 0.18 ± 0.09 vs 0.06 ± 0.0, n = 8–10, p < 0.001) and Psd-95 expression (C57 vs A IPOe KO, Contralateral, 0.10 ± 0.008 vs 0.078 ± 0.004; Ipsilateral 0.099 ± 0.006 vs 0.064 ± 0.008, n = 8–10, p < 0.05) (Fig. 8C, D). Daidzein treatment further reduced synaptophysin and Psd-95 gene expression in A IPOe KO mice (Fig. 8C, D). Synaptophysin and Psd-95 mRNA levels were significantly correlated in both hemispheres regardless of treatment (Fig. 8G, H). Unlike C57 mice shown in Figure 8F, I, the daidzein-induced selective elevation of synaptophysin gene and protein in the stroke-hemisphere was absent in ApoE deficiency (Fig. 8H, J). Together, the results showed that ApoE is necessary for daidzein-induced synaptophysin expression in chronic stroke.

**Discussion**

Injury-induced repair and remodeling occur during critical periods following stroke. These processes provide a temporal window in which augmentation of molecular and synaptic changes can lead to behavioral adaptation/recovery. The current work identified daidzein as an enhancer of the genetic program governing
cholesterol homeostasis and a potential stroke recovery agent. The daidzein-induced benefits include increased expression of genes that regulate the cholesterol homeostasis both in cultures and poststroke brain, and enhanced motor/gait function during the chronic recovery phase. Notably, the study revealed that daidzein-induced ApoE upregulation is essential for enhancing motor/gait functions and upregulation of synaptophysin, a presynaptic element.

Literature shows that isoflavones, including daidzein, inhibits HMG-CoA reductase, an enzyme for cholesterol biosynthesis (Sung et al., 2004; Jung and Kim, 2013). Because daidzein was known to activate PPARγ, an upstream transcription factor of Lxr, the study focused on the effect of daidzein on expression of the genes involved in cholesterol homeostasis rather than its biosynthetic pathway. Not only Lxr and the downstream target transporters were elevated in vitro, daidzein also elevated the cholesterol homeostasis and a potential stroke recovery agent.
Table 2. Gene expression in the brain 1 month after strokea

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<tr>
<th></th>
<th>Vehicle Contralateral</th>
<th>Vehicle Ipsilateral</th>
<th>Daidzein Contralateral</th>
<th>Daidzein Ipsilateral</th>
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<td>Ppar γ</td>
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<td>1.43E-03 ± 1.1E-04</td>
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<td>8.04E-03 ± 5.7E-04</td>
<td>5.34E-03 ± 4.4E-04</td>
<td>13.41E-03 ± 21.1E-04</td>
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<td>Apoe</td>
<td>7.87E-01 ± 6.4E-02</td>
<td>11.65E-01 ± 5.4E-02</td>
<td>8.17E-01 ± 3.2E-02</td>
<td>17.12E-01 ± 21.2E-02</td>
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<td>Lp1</td>
<td>4.22E-02 ± 4.1E-03</td>
<td>4.76E-02 ± 3.8E-03</td>
<td>4.02E-02 ± 3.1E-03</td>
<td>5.76E-02 ± 4.4E-03</td>
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<td>1.84E-01 ± 1.1E-02</td>
<td>1.82E-01 ± 0.9E-02</td>
<td>2.38E-01 ± 1.1E-02</td>
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<td>1.67E-01 ± 9.1E-03</td>
<td>1.79E-01 ± 4.8E-03</td>
<td>1.40E-01 ± 13.3E-03</td>
<td>1.36E-01 ± 5.4E-03</td>
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<td>Psd-95</td>
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<td>0.99E-01 ± 6.8E-03</td>
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<td>8.07E-02 ± 7.4E-03</td>
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<td>Fas</td>
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<td>2.19E-02 ± 1.7E-03</td>
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<td>Lpl</td>
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<td>1.11E-02 ± 2.2E-03</td>
<td>1.01E-02 ± 7.3E-04</td>
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<td>Sreb1p</td>
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<td>1.10E-03 ± 10.1E-05</td>
<td>1.00E-03 ± 5.2E-05</td>
<td>1.45E-03 ± 9.6E-05</td>
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</table>

aData are mean ± SEM. →, ↑, and ↓ indicate no and significant gene changes in the ipsilateral hemisphere by daidzein treatment.

*b < 0.05 versus contralateral (two-way ANOVA and post hoc Newman-Keuls test); n = 8–11.

*p < 0.05 versus ipsilateral of vehicle (two-way ANOVA and post hoc Newman-Keuls test); n = 8–11.

**p < 0.05 versus contralateral of vehicle (two-way ANOVA and post hoc Newman-Keuls test); n = 8–11.

Figure 5. Daidzein reduces stroke-induced body weight loss and improves behaviors. Longitudinal body weight measurement and behavior test were performed in vehicle (Veh) or daidzein (Dz)-treated C57 mice during acute and recovery phases. A, Percentage changes of body weight following ischemia. The stroke-induced body weight reduction was attenuated in daidzein-treated mice during a critical period (3–6 d after stroke); n = 22. *p < 0.05 versus Veh. B, Rotated performance before (pre) and during the 4 week (w) postschismic period, latency to fall measured in seconds; n = 9–11/group. C, D, Catwalk gait analyses: walk speed, the average speed expressed in distance units per second (C). Regularity index, a parameter to gauge the degree of interlimb coordination (D). All behavior results are presented as percentage of preischemic baseline (% Pre, mean ± SEM); n = 22 or 23/group. *p < 0.05 (two-way ANOVA with post hoc Bonferroni tests). **p < 0.01 (two-way ANOVA with post hoc Bonferroni tests). ##p < 0.01, Veh versus Dz (effect of treatment) (two-way ANOVA with post hoc Bonferroni tests). *p < 0.05, Veh versus Dz (effect of treatment) (two-way ANOVA with post hoc Bonferroni tests).

LXR-directed approach stimulates transcription of Sreb1p and its target genes Fas and Lpl. FAS and LPL are linked to the synthesis of fatty acids and triglycerides, resulting in hypertriglyceridemia and hepatic steatosis (Grefhorst et al., 2002; Chisholm et al., 2003). Thus, an optimal therapeutic strategy would be to selectively activate cholesterol efflux pathways without stimulating...
genes linked to lipogenesis (Kratzer et al., 2009). Compared with T0901317, which upregulated Srebp1, Fas, and Lpl transcription in primary astrocytes, daidzein increased Srebp1 transcription without affecting the induction of Lpl and Fas genes in vitro (Fig. 1) and in the postischemic brain (Table 2). These findings suggest a potential advantage of daidzein over T0901317 for chronic use in stroke recovery.

The daidzein-induced functional benefits occurred in the absence of neuroprotection. Our previous in vitro data and literature suggested that daidzein, which has years of use in humans, could possibly be neuroprotective and neurorestorative (Ma et al., 2010; Hurtado et al., 2012). However, our current detailed in vivo studies showed that while daidzein enhanced functional recovery, it neither increased nor decreased infarct size, consistent with a study in rats (Stout et al., 2013). The dissociation of functional recovery from neuroprotection indicates the presence of repair process mechanisms that may be distinct from mechanisms that underlie acute pathology/protection. The stage-specific mechanisms reflect the anatomical distinction of the tissues involved in different stages of chronic stroke. While acute pathology involves the ipsilateral hemisphere, functional recovery is thought to require plasticity from noninjured tissues around the infarct and the contralateral hemisphere (Zeiler et al., 2013; Qin et al., 2014). It is noteworthy that the same drug and/or target may respond differently depending on the context of poststroke stages. For example, whereas an agent that inhibits stroke-induced excessive tonic inhibition (net effect of excitation) enhances stroke recovery, the treatment, if given too early (i.e., <3 d after stroke), exacerbates acute stroke injury (Clarkson et al., 2010). Thus, agents such as daidzein would allow treatment at an early poststroke stage and provide a wider treatment window for subsequent functional enhancement.

In the current study, daidzein treatment was initiated at the early reperfusion period and continuously administered for 1 month. Several pharmacokinetic studies of chronic daidzein treatment in both humans and rodents have shown increased concentration of isoflavones in body fluids and tissues. With a half-life of ~6 h in humans and 12 h in rodents (King and Bursill, 1998; Coldham and Sauer, 2000), chronic daidzein administration was shown to be safe in clinical trials at a dose of 0.5–1 mg/kg/d for 6 months (NCT00951912) or 12 months (NCT01463436). Although the dose used in this study in mice was higher (5–10 mg/kg/d) and the treatment duration was shorter (1 month) than that used in the above clinical studies, it was within the range of other rodent studies (0.1–50 mg/kg/d) (Rivera et al., 2013; Soumyakrishnan et al., 2014). Importantly, we found that chronic daidzein administration did not increase injury size and hemispheric swelling (Figs. 3F, 4G). The absence of the adverse stroke outcomes in mice together with no evident toxicity in cultures (Fig. 1P) supports safety of daidzein for early and long-term use in chronic stroke.

Analysis of the motor/gait function in C57 mice revealed two sets of behaviors that spontaneously recovered and ones with sustained impairments (Figs. 5, 6). Daidzein improved the behaviors that showed sustained deficits (e.g., rotarod, speed, stride length, and swing speed). On the other hand, the literature indicates that unilateral stroke induces deficits in the limbs of both sides, which has been described in rodent and human (Schafer et al., 2009; Darling et al., 2011; Pandian and Arya, 2013; Qin et al., 2014). The observations suggest that the ipsilesional side was not “nonaffected” but rather “less affected.” The underlying mechanism that accounts for the acute bilateral deficits may include plastic changes in the intact hemisphere and disturbed interhemispheric connectivity, probably due to the ongoing injury in the affected hemisphere that might be involved in the functional impairments in the unaffected limbs (Gonzalez et al., 2004; van Meer et al., 2010). Individual gait assessment in this study revealed that the stroke-induced bilateral deficits and daidzein treatment resulted in bilateral enhancement (Fig. 6). Because animals were subjected to unilateral right MCAO, improved function in the less affected limb (e.g., RH) suggests a potential role of the contralesional hemisphere in functional recovery (Qin et al., 2014). Relevantly, we observed that daidzein increased Apoe mRNA at 3 d in the contralateral hemisphere (Fig. 3E; Table 1), suggesting an intriguing possibility that this early rise in Apoe
mRNA in the contralateral hemisphere may cause subsequent benefits in stroke recovery. There is no reported deficit in neurocognitive and retinal function in a human subject lacking functional APOE gene expression (Mak et al., 2014). In agreement with this clinical finding, ApoE deficiency in mice did not affect baseline behaviors in this study (preischemic rotarod C57 vs Apoe KO, 253 ± 49.4 s, 222.8 ± 48.1 s, n = 11/group. C, D. Catwalk gait analyses: Walk speed, the average speed expressed in distance units per second (C). Regularity index, a parameter to gauge the degree of interlimb coordination (D). E–G. Catwalk gait parameters for individual limb in LF, LH, RF, and RH.Stride length (E), swing speed (F), and mean intensity (G). All behavior results are presented as percentage of preischemic baseline (% Pre, mean ± SEM); n = 10–13/group for gait analysis. *p < 0.05 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). **p < 0.01 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). ***p < 0.001 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). #p < 0.05, Veh versus Dz (effect of treatment) (two-way ANOVA with post hoc Bonferroni tests). H–J. mRNA levels in the 1 month poststroke brain of Apoe KO mice, Lxr (H), Abca1 (I), and Abcg1 (J); n = 10–13/group. *p < 0.05 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). **p < 0.01 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). ***p < 0.001 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). #p < 0.05 versus Veh ipsilateral (two-way ANOVA and post hoc Newman–Keuls test). **p < 0.01 versus Veh ipsilateral (two-way ANOVA and post hoc Newman–Keuls test).

Because of its reduced capacity for lipidation and efflux of cholesterol and phospholipids, APOEe4 has been linked to a high risk of developing Alzheimer’s disease (AD) (Michikawa et al., 2000; Hanson et al., 2013). Because ApoE expression level is a risk factor for AD regardless of APOEe4 allele status (Maloney et al., 2010), enhancing ApoE and other cholesterol transporter expression by activating LXRα and RXRα has been suggested as a way to improve AD pathology (Mandrekar-Colucci and Landreth, 2011; Cramer et al., 2012; Boehm-Cagan and Michaelson, 2014). The current study provides the mechanistic evidence of ApoE for daidzein-induced functional benefits and synaptophysin upregulation. Of particular interest, in light of our functional daidzein results is that APOEe4 presence predicted age-related gait speed decline in men (Verghese et al., 2013). Likewise, the presence of the APOEe4 allele is associated with reduced short- and long-term recovery from stroke (Cramer and Procaccio, 2012). Our working model is daidzein-induced upregulation of Lxr and

Figure 7. Daidzein-induced functional benefits are absent in ApoE deficiency. Longitudinal behavior test results in vehicle (veh) and daidzein (Dz)-treated Apoe KO mice during acute and chronic recovery phases. A, Percentage changes of body weight following ischemia. Note the similar weight reduction in both groups during a critical period (3–6 d poststroke); n = 10–12/group. B, Rotarod performance before (pre) and during the postischemic period up to 4 weeks (w), latency to fall measured in seconds; n = 11/group. C, D, Catwalk gait analyses: Walk speed, the average speed expressed in distance units per second (C). Regularity index, a parameter to gauge the degree of interlimb coordination (D). E–G, Catwalk gait parameters for individual limb in LF, LH, RF, and RH. Stride length (E), swing speed (F), and mean intensity (G). All behavior results are presented as percentage of preischemic baseline (% Pre, mean ± SEM); n = 10–13/group for gait analysis. *p < 0.05 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). **p < 0.01 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). ***p < 0.001 versus Pre (effect of stroke) (two-way ANOVA with post hoc Bonferroni tests). #p < 0.05, Veh versus Dz (effect of treatment) (two-way ANOVA with post hoc Bonferroni tests). H–J, mRNA levels in the 1 month poststroke brain of Apoe KO mice, Lxr (H), Abca1 (I), and Abcg1 (J); n = 10–13/group. *p < 0.05 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). **p < 0.01 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). ***p < 0.001 versus contralateral side (two-way ANOVA and post hoc Newman–Keuls test). #p < 0.05 versus Veh ipsilateral (two-way ANOVA and post hoc Newman–Keuls test). **p < 0.01 versus Veh ipsilateral (two-way ANOVA and post hoc Newman–Keuls test).
transporter genes provide a larger substrate base for conventional cholesterol effusion.

The required ApoE for daidzein-induced synaptophysin upregulation suggests mechanisms for cholesterol uptake into neurons. Low-density lipoprotein-related receptor protein-1 (LRP1) is an ApoE receptor that involves receptor-mediated endocytosis for cholesterol transport. Notably, we found that Lrp1 mRNA level in the brain was high in the ipsilateral hemisphere at 1 month after stroke (H1101110-fold higher than low-density lipoprotein receptor gene Ldlr; Table 2). Correlation analyses showed that stroke-induced Lrp1 expression is significantly correlated with Apoe \((r^2 = 0.399, p < 0.05, n = 8)\) and synaptophysin mRNA \((r^2 = 0.432, p < 0.05, n = 8)\) in a daidzein-treated group, but not in a vehicle-treated group. Although there was no significant correlation between Apoe and synaptophysin mRNA levels, the correlation slope in daidzein-treated group was significantly elevated \((p < 0.05)\). Therefore, it is unlikely that ApoE directly regulates synaptophysin transcription in the presence of daidzein, but rather through LRP1 for ApoE uptake for synaptophysin expression. Thus, daidzein is likely promoting the directional movement of cholesterol through LRP1 in the injured brain for neurite outgrowth for plastic changes.

Through the gain and loss of ApoE coupled with molecular and functional studies, the current study demonstrated that daidzein-induced ApoE is critical for functional recovery in chronic stroke. The caveat of the study is a need to provide relevance of the preclinical findings in mice to human patients. Nevertheless, the similarities in the anatomical organization of lumbar pattern generators and locomotor control mechanisms between humans and rodents (Gerasimenko et al., 2008) suggest shared mechanisms in locomotor/kinematic recovery following stroke. As walking speed is one of the best determinants of community ambulation and of high priority to patients in the immediate aftermath of a paralytic stroke, our findings of better locomotion, faster gait speed, larger stride length and swing speed at the system levels, together with elevated ApoE and synaptophysin expression at the cellular level, provide a biological rationale for strategies aimed at augmenting ApoE for stroke recovery. With its known safety in humans, early and chronic use of clinically approved daidzein to aim at ApoE upregulation without having adverse effect on infarct size may serve as a novel, translatable strategy to promote recovery in stroke patients. Moreover, the findings should serve to catalyze additional studies on the association...
of prevalent ApoE isoforms with outcomes following stroke in humans.

References


