Recent findings suggest that neurotoxicity is the mechanism underlying the induction of neuronal insulin resistance by a high cholesterol diet. Troxerutin, a naturally occurring flavonoid, has been reported to possess biological activity beneficial to human health. Our recent studies have demonstrated that troxerutin attenuates cognitive impairment and oxidative stress induced by D-galactose in mouse brain through decreasing advanced glycation end products, reactive oxygen species and protein carbonyl levels and enhancing phosphoinositide 3-kinase/Akt activation. In this study, we evaluated the effect of troxerutin on cognitive impairment induced by brain insulin resistance in mice fed a high-cholesterol diet, and explored its potential mechanism. Our results showed that oral administration of troxerutin to these mice significantly improved behavioural performance in a step-through passive avoidance task and a Morris water maze task, at least in part, by decreasing the levels of reactive oxygen species, protein carbonyl and advanced glycation end products and blocking endoplasmic reticulum stress via reduced phosphorylation of the pancreatic endoplasmic reticulum-resident kinase and eukaryotic translation initiation factor 2α. Furthermore, troxerutin significantly inhibited the activation of c-jun N-terminal kinase and IκB kinase β/nuclear factor-κB induced by endoplasmic reticulum stress and enhanced insulin signalling pathway, which prevented obesity, restored normal levels of blood glucose, fatty acids and cholesterol and increased the phosphorylation of cyclic adenosine monophosphate response element-binding protein and the expression levels of c-fos in the hippocampus. Moreover, troxerutin significantly inhibited endoplasmic reticulum stress-induced apoptosis and decreased the activation of caspase-12 and caspase-3, and reduced the mean optical density of the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end label-positive cells in the hippocampus. However, intra-cerebroventricular infusion of PI-103, a specific phosphoinositide 3-kinase 110α inhibitor, significantly inhibited the expression levels of phosphoinositide 3-kinase 110α and phosphoinositide 3-kinase downstream signalling in the hippocampus of mice co-treated with high cholesterol and troxerutin and vehicle control mice. These results suggest that troxerutin could be recommended as a possible candidate for the prevention and therapy of cognitive deficits in type 2 diabetes mellitus and Alzheimer’s disease.

**Keywords:** troxerutin; high-cholesterol diet; cognitive deficits; phosphoinositide 3-kinase/Akt/cAMP response element-binding protein pathway; endoplasmic reticulum stress
Introduction

Cholesterol is an essential component of brain and nerve cells and plays an important role in maintaining the function of the nervous system. There is increasing evidence that a high-cholesterol diet can induce neuroinflammation and neuroapoptosis, ultimately resulting in neurodegeneration and cognitive deficits (Puglielli et al., 2003; Casserly and Topol, 2004; Lu et al., 2006a, 2009). Recent findings suggest that neuronal insulin resistance induced by high-dietary cholesterol is the underlying mechanism (Taghibiglou et al., 2009). There is a substantial amount of experimental and clinical evidence that brain insulin resistance and insulin deficiency are implicated in the pathogenesis of cognitive impairment and neurodegeneration, particularly Alzheimer's disease (Sinclair et al., 2000; Schubert et al., 2004; Craft, 2005; Stranahan et al., 2008a, b; de la Monte, 2009; Marks et al., 2009; Moloney et al., 2010).

Troxerutin, a trihydroxethylated derivative of the natural bioflavonoid rutin, has been reported to possess important biological activity, including antioxidative and anti-inflammatory activity (Blasig et al., 1987, 1988; Fan et al., 2009; Zhang et al., 2009). Our recent reports confirm the neuroprotective effects of troxerutin against oxidative damage induced by D-galactose (Lu et al., 2010b, d). In addition, our findings indicate that troxerutin inhibits the formation of advanced glycation end products in vivo and enhances insulin signalling (Lu et al., 2010b, d). Advanced glycation end products are a harmful post-translational protein modification and a trigger of excess reactive oxygen species and abnormally high oxidative stress that all contribute to the early phases of age-related disease such as diabetes, atherosclerosis and Alzheimer’s disease (Tian et al., 2005). Emerging evidence demonstrates that a high-cholesterol diet induces oxidative stress and deposition of advanced glycation end products that will cause endoplasmic reticulum stress, impair insulin sensitivity and ultimately lead to diabetes mellitus (Sandu et al., 2005; Tokita et al., 2005; Liu et al., 2010; Sato et al., 2010). There is no report in the literature of studies designed to investigate whether troxerutin has a protective effect against cognitive impairment induced by brain insulin resistance in mice fed a high-cholesterol diet. This study is designed to address this issue and investigate the potential mechanism underlying its action.

Materials and methods

Animals and administration

Sixty 4-week-old male C57BL/6 strain mice were purchased from the Branch of National Breeder Centre of Rodents (Shanghai). Before the experimental stage, the mice had free access to food and water ad libitum and were kept at 23 ± 1°C and 60% relative humidity. After acclimatization to these conditions for 1 week, mice were randomly divided into six groups of 10 mice and kept on a 12 h light/12 h dark cycle. Each group received one of the following treatments for 20 weeks:

Vehicle group: normal chow, daily intra-cerebroventricular infusion of vehicle (PI-103 solvent: 99% sterile saline, 1% dimethyl sulphoxide), daily oral administration of sterile distilled water containing the troxerutin solvent (0.1% v/v Tween-80) after 0.5 h of intra-cerebroventricular infusion.

High-cholesterol diet group: normal chow plus 5% cholesterol, daily intra-cerebroventricular infusion of PI-103 solvent, daily oral administration of troxerutin solvent after 0.5 h of intra-cerebroventricular infusion.

High-cholesterol/troxerutin group: normal chow plus 5% cholesterol, daily intra-cerebroventricular infusion of the PI-103 solvent, daily oral administration of troxerutin at doses of 150 mg/kg/day after 0.5 h of intra-cerebroventricular infusion.

High-cholesterol/PI-103/troxerutin group: normal chow plus 5% cholesterol, daily intra-cerebroventricular infusion of PI-103 solvent, daily oral administration of troxerutin at doses of 150 mg/kg/day after 0.5 h of intra-cerebroventricular infusion.

The daily infusion of PI-103 (0.1 µg/µl) was done with a microinjector (KD scientific Inc., Holliston, MA, USA) through both cerebral ventricles (from the bregma: anteroposterior, 0.5 mm; mediolateral, 1.0 mm; depth, 2.0 mm) at a rate of 2.5 µl/min for 20 weeks (Paxinos and Franklin, 2001). The drug dosage and period used in this study were based on earlier reports and the results of our pilot study (data not shown here) (Lu et al., 2006a, 2009, 2010b, d). All mouse experiments were in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the respective university committees for animal experiments. After the behavioural testing, mice were sacrificed and brain tissue was collected immediately for experiments or stored at -70°C.

Behavioural tests

After 19 weeks on a high-cholesterol diet, all behavioural tests were done in the 20th week.

Step-through passive avoidance task

The step-through passive avoidance apparatus consisted of an illuminated chamber (11.5 cm × 9.5 cm × 11 cm) lit with a 25 W lamp,
attached to a darkened chamber (23.5 cm × 9.5 cm × 11 cm) containing a metal floor that could deliver a mild electric shock (0.3 mA, 50 Hz, 5 s). A guillotine door separated the two compartments. The step-through test was done as previously described (Lu et al., 2006b). Briefly, mice were placed in a dimly lit room containing the apparatus for 0.5 h before training, to acclimate to the new environment. Each mouse was placed into the illuminated chamber, facing away from the door to the dark chamber and allowed to acclimate for 1 min. As soon as the mouse entered the dark chamber the door was slid back into place, triggering an electric shock. The mouse was immediately removed from the chamber and returned to its cage. The latency (time used to change compartment) was recorded. The retention test was conducted 24 h later with the mouse again being placed in the illuminated chamber and subjected to the same protocol but without the electric shock. The upper time limit was set at 300 s.

Morris water maze test
The Morris water maze test was done as described (Lu et al., 2009). The experimental apparatus consisted of a circular water tank (100 cm diameter, 35 cm height), containing water to a depth of 15.5 cm. The water was controlled at 23 ± 1°C and was rendered opaque by adding powdered milk. A platform of 4.5 cm in diameter and 14.5 cm height, so that the platform was 1 cm below the water surface, was placed at the midpoint of one quadrant. The pool was located in a test room that contained various prominent visual cues. Each mouse received four training periods on each of four consecutive days. Latency to escape from the water maze (finding the submerged escape platform) was calculated for each trial. On Day 5, the probe test was carried out by removing the platform and allowing each mouse to swim freely for 60 s. The time that mice spent swimming in the target quadrant (where the platform was located during the hidden platform training) was measured. For the probe trials, the number of times the mouse crossed over the platform site was recorded. All data were recorded with a computerized video system.

Tissue homogenates
After 20 weeks on a high-cholesterol diet, mice were deeply anaesthetized and sacrificed. For biochemical studies as described (Lu et al., 2010b, d), the hippocampus was promptly dissected and homogenized in 1:5 (w/v) 50 mM (pH 7.4) ice-cold Tris-buffered saline containing a protease inhibitor cocktail (Sigma-Aldrich, MO, USA) with 10 strokes at 1200 rpm in a Teflon-glass Potter-Elvehjem homogenizer (Kontes, Vineland, NJ, USA). Homogenates were centrifuged at 12,000 g for 10 min and the supernatant was used to determine hippocampal advanced glycation end products, reactive oxygen species and protein carbonyl levels.

For western blot analysis as described previously (Lu et al., 2006a, 2009, 2010b, d), the hippocampus was homogenized in 1:3 (w/v) ice-cold radioimmunoprecipitation assay lysis buffer (Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 0.004% sodium azide) supplemented with 30 μl of 10 mg/ml phenylmethylsulphonyl fluoride, 30 μl of Na3VO4, 30 μl of Na2 and 30 μl of protease inhibitor cocktail per 1 g of tissue. The homogenates were sonicated four times for 30 s with 20 s intervals using a sonicator, centrifuged at 15,000 g for 10 min at 4°C, then the supernatant collected and stored at −20°C for western blot studies. Nuclear factor-κB (NF-κB) p65 expression was determined by western blot assessment in cytoplasmic and nuclear extracts of brain tissues that were obtained using a nuclear/cytoplasmic isolation kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein levels in the supernatants were determined using the biocinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Body weight and biochemical analysis
Body weight was measured every week after 6 h fasting. After 19 weeks on a high-cholesterol diet, a glucose tolerance test were done by intraperitoneal glucose injection (2 g/kg) following a 6 h food withdrawal. Immediately before and at 15, 30, 60, 90 and 120 min after glucose administration, blood glucose levels were measured with an Ascensia Elite glucose meter (Bayer Corporation, Mishawaka, IN, USA). For glucose/insulin measurements, blood samples were taken by tail venipuncture as described (Miller et al., 2005). For insulin measurements, whole blood was centrifuged at 16,000 g for 7 min to pellet blood cells. The plasma was transferred to a fresh tube and placed on dry ice, after which it was stored at −80°C. Serum insulin and leptin levels were measured with the appropriate enzyme-linked immunosorbent assay kits (ALPCO Diagnostics, Windham, NH, USA).

Determination of blood free fatty acids, triglycerides and cholesterol
Plasma levels of non-esterified fatty acids were measured using a non-esterified fatty acids assay C kit (Wako Chemicals, Richmond, VA, USA) according to the manufacturer’s instructions. Triglycerides and total cholesterol were measured directly in whole blood using a Cardiochek® meter (PTS, Indianapolis, IL, USA).

Advanced glycation end products enzyme-linked immunosorbent assay
Quantitative measurement of advanced glycation end products was done by advanced glycation end products enzyme-linked immunosorbent assay as described previously (Lu et al., 2010c, d). Briefly, 96-well plates were coated with 100 μl/well of 3 μg/ml advanced glycation end products bovine serum albumin in coating buffer overnight at 4°C. Wells were washed three times with 150 μl washing buffer (phosphate buffered saline, 0.05% Tween 20, 1 mM NaNNa3) then blocked with 1% normal goat serum in 100 μl 100 μl of phosphate buffered saline for 2 h at 37°C. After washing, 50 μl of 1:10 (v/v) diluted samples in dilution buffer (phosphate buffered saline, 0.02% Tween 20, 1 mM NaNNa3, 1% normal goat serum) and 50 μl of anti-advanced glycation end products polyclonal antiserum in coating buffer overnight at 4°C were added. Plates were incubated at room temperature for 2 h with gentle agitation on a horizontal rotary shaker. Wells were washed and 100 μl of alkaline phosphate conjugated secondary antibody in dilution buffer (1:2000) was added. The plates were incubated at 37°C for 1 h then washed six times as described (above) and 100 μl of p-nitrophenyl phosphate substrate was added to each well. After 60 min, optical density at 405 nm was determined with a microplate fluorometer FlexStation II (Molecular Devices, San Francisco, CA, USA). The advanced glycation end products-BSA was used as a competing antigen to generate an advanced glycation end products standard curve. Sample advanced glycation end products values were calculated from the standard curve.

Reactive oxygen species assay
Reactive oxygen species was measured as described based on the oxidation of 2’,7’-dichlorofluorescein diacetate to

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2′,7′-dichlorofluorescein (Lu et al., 2010b, c, d). Briefly, the homogenate was diluted 1:20 (v/v) with ice-cold Locke’s buffer [154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 2.0 mM CaCl2, 10 mM D-glucose and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4] to obtain a tissue concentration of 10 mg/ml. The reaction mixture (1 ml) containing Locke’s buffer, 0.2 ml of homogenate and 10 ml of 5 mM 2′,7′-dichlorodihydrofluorescein diacetate was incubated for 15 min at room temperature to allow the 2′,7′-dichlorodihydrofluorescein diacetate to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After 30 min of further incubation, the conversion of 2′,7′-dichlorodihydrofluorescein diacetate to the fluorescent product 2′,7′-dichlorofluorescein was measured using a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of 2′,7′-dichlorodihydrofluorescein diacetate in the absence of homogenate) was corrected by the inclusion of parallel blanks. Reactive oxygen species formation was quantified from a 2′,7′-dichlorofluorescein standard curve and the data are expressed as pmol 2′,7′-dichlorofluorescein formed/min/mg protein.

Oxidative stress marker: protein carbonyls

Protein carbonyl content was measured as a marker of oxidative damage to proteins (Lu et al., 2010c). The hydrazone derivatives were determined between 360 and 390 nm (ε = 21.0 mM/cm) as described (Lu et al., 2010c). Data are expressed as nmol carbonyls/mg protein.

Immunoprecipitation assay and western blot analysis

The immunoprecipitation assay was previously described (Lu et al., 2010d). The brain was homogenized in 1:3 (v/v) ice-cold lysis buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4, 125 mM NaCl, 25 mM NaF, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Nonidet™ P-40, 1 mM Na2VO4 and the protease inhibitor mixture]. Equal amounts of protein (500 μg) were cleared using protein A-Sepharose for 1 h at 4°C and then incubated with 2–3 μg of antibody against the insulin receptor substrate 1 (IRS1) (Cell Signalling Technology, Inc., Beverly, MA, USA). The immune complexes were affinity-precipitated with protein A-Sepharose beads and washed six times with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, 10 mM MgCl2, 1 mM NaF, 1% Nonidet™ P-40 and 1 mM Na2VO4. The immune complexes were then submitted to denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis and analysed by western blot with rabbit polyclonal anti-phospho-IRS1 (Ser307) (1:1000; Cell Signalling Technology, Inc., Beverly, MA, USA), rabbit polyclonal anti-Pi3K p85α (1:1000; Cell Signalling Technology, Inc., Beverly, MA, USA) and rabbit polyclonal anti-IRS1.

The protein samples (80 μg) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) by electrophoretic transfer. The membrane was blocked with 5% non-fat milk and 0.1% Tween-20 in Tris-buffered saline and incubated overnight with primary antibodies including mouse anti-NF-kB p65 (Santa Cruz Biotechnology, CA, USA); rabbit anti-phospho-pancreatic endoplasmic reticulum-resident kinase (PERK) (Thr980), rabbit anti-phospho-eukaryotic translation initiation factor 2α (eIF2α) (Ser51), rabbit anti-phospho-c-jun N-terminal kinase (JNK) (Thr183/Tyr185), rabbit anti-phospho-ixB kinase β (IkKβ) (Ser181), rabbit anti-phospho-Akt (Ser473), mouse anti-phospho-cAMP response element-binding protein (CREB) (Ser133) and rabbit anti-ixBζ, anti-c-Fox, anti-cleaved caspase-3 and anti-caspase-12 (Cell Signalling Technology, Inc., Beverly, MA, USA). The proteins were detected using horseradish peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse secondary antibodies. The optical density values of bands were measured with Scion Image analysis software (Scion Corp., Frederick, MD, USA) and were calculated from a mixture of protein and antibody (diluted 1:1000; Cell Signalling Technology, Inc., Beverly, MA, USA) and anti-JNK1 (Abcam, Cambridge, UK) as an internal control (optical density detected protein/optical density internal control).

Collection of brain slices

The mice were perfused transcardially with 25 ml of normal saline [0.9% (w/v) NaCl]. The brain tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) or acetic acid at 4°C for 4 h, incubated overnight at 4°C in 100 mM sodium phosphate buffer (pH 7.4) containing 30% sucrose; and embedded in Optimal Cutting Temperature media (Leica Microsystems, Nussloch, Germany). Coronal sections (12 μm) from cryofixed tissue were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma-Aldrich, MO, USA) and stored at –70°C.

Immunohistochemistry

For immunohistochemistry, endogenous peroxidase activity in the sectioned tissues was blocked with 3% H2O2, and non-specific binding sites were blocked with 3% normal goat serum (Chemicon International Inc., Temecula, CA, USA). The sections were incubated at 4°C overnight with rabbit anti-Pi3K p85α (1:100, Millipore, Billerica, MA, USA) in Tris-buffered saline containing 1% goat serum, respectively. Subsequently, biotinylated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (diluted as described by supplier; Vector Laboratories, Inc., Burlingame, CA, USA) was applied, followed by incubation for 1 h with an avidin–biotin–horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories, Inc.). Horseradish peroxidase was reacted with diaminobenzidine and H2O2 for 5 min to yield a permanent deposit. Stained whole mounts were rinsed in distilled water, mounted on 3-aminopropyl-trimethoxysilane-coated slides, air-dried overnight, dehydrated in ethanol, cleared in xylene and covered with Cytoseal (Stephens Scientific, Kalamazoo, MI, USA). The specificity of the staining was assessed by omitting the primary antibody.

Stained specimens from 30 mice (five from each group) were captured using a Zeiss Axioskop 40 microscope (×10 or ×40 objective) (Carl Zeiss, Oberkochen, Germany) and the images were taken with a charge-coupled device camera (COOLSNAP Color, Photometrics, Roper Scientific, Inc.). To assess the intensity of immunoreactivity, levels of staining were quantified using Image Pro-Plus 6.0 software (Media Cybernetics Inc., USA). Values (three slides for each brain) of optical density in individual cells represented quantities of objective proteins, and were calculated by the equation: \[ \sum_{	ext{area}} \frac{\text{integral optical density}}{\text{integral optical density}} \] where integral optical density is the integral optical density in a region of interest, and area is the area of a region of interest. In this study, a region of interest represents an individual cell. \[ \sum_{	ext{area}} \text{Area} \] is the total area of all cells in the photograph and \[ \sum_{	ext{area}} \text{Area} \] is the total area of all cells in the photograph.
density is the sum of integral optical density of all cells in the photograph (Xavier et al., 2005).

Immunofluorescence staining

Immunofluorescence staining or double immunostaining was done on cryofixed sections cut and mounted as described above. The double staining was done by firstly adding primary antibodies, followed by incubation at 4°C overnight. The following antibody was used: rabbit anti-phospho-INK (Thr183/Tyr185) (1:350; Cell Signalling Technology, Inc., Beverly, MA, USA). The section was washed in phosphate buffered saline and then secondary antibody (Cy3-conjugated anti-rabbit 1:150, Abcam, Cambridge, UK) was applied. The specificity of the staining was assessed by omitting the primary antibody. Sections were counterstained with ProLong® Gold containing 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Stained specimens from 30 mice (five from each group) were captured using a Zeiss Axioskop 40 microscope (×10 or ×40 objective) (Carl Zeiss, Oberkochen, Germany) and the images taken with a charge-coupled device camera (CoolSNAP Color, Photometrics, Roper Scientific, Inc). For quantitative analysis of the immunofluorescence staining, values of integral optical density and region of interest were measured by Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA). Plaque areas were excluded and values (three slides for each brain) were calculated as described above.

Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling assay

The standard protocol for frozen sections was followed for the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end (TUNEL) staining (BD ApoAlert® DNA fragmentation assay kit, BD Biosciences Clontech, Palo Alto, CA, USA). The sections were immersed in a Coplin jar containing fresh 4% formaldehyde in phosphate buffered saline and then incubated at room temperature for 5 minutes. The sections were washed twice with phosphate buffered saline (5 min each wash). The liquid was allowed to drain thoroughly and the slides were placed onto a flat surface. Each section was covered with 100 μl of 20 μg/ml proteinase K solution and incubated at 37°C for 5 min.

After two washes with phosphate buffered saline (5 min each wash), the sections were transferred into a Coplin jar containing 4% formaldehyde in phosphate buffered saline and then washed in phosphate buffered saline again. The cells were covered in equilibration buffer (from the kit) and kept at room temperature for 5 min to equilibrate. The equilibration buffer was drained off, and TdT incubation buffer was added to the tissue sections. The slice was placed in darkness in a humidified 37°C incubator for 1 hr for the tailing reaction, which was terminated by immersing the samples in 2× standard saline citrate and kept at room temperature for 15 min. Samples were washed three times with phosphate buffered saline (5 min each wash) to remove unincorporated fluorescein-dUTP. Finally, strong, nuclear green fluorescence in apoptotic cells was observed with a fluorescence microscope equipped with a standard fluorescein filter (520 ± 20 nm). All cells stained with propidium iodide exhibited strong red cytoplasmic fluorescence when viewed under light of 620 nm wavelength. Stained specimens from the 30 mice were captured using a Zeiss Axioskop 40 microscope (×10 or ×40 objective) (Carl Zeiss, Oberkochen, Germany) and the images were taken with a charge-coupled device camera (CoolSNAP Color, Photometrics, Roper Scientific, Inc). For quantitative analysis of the immunofluorescence staining, values of integral optical density and region of interest were measured by Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA). Plaque areas were excluded and values (three slides for each brain) were calculated as described above.

Statistical analysis

All statistical analysis was done with SPSS software, version 11.5. Group differences in the escape latency in the Morris water maze training task were analysed using two-way ANOVA with repeated measures, the factors being treatment and training day. The other data were analysed with one-way ANOVA followed by Newman–Keuls or Tukey’s Honestly Significant Difference post hoc test. Data are expressed as mean ± SD. Statistical significance was set at P < 0.05 for all tests.

Results

Troxerutin blocks the endoplasmic reticulum stress pathway through decreasing the levels of reactive oxygen species, protein carbonyl and advanced glycation end products in the hippocampus of mice fed a high-cholesterol diet

Figure 1 shows that a high-cholesterol diet resulted in a significant increase in the levels of reactive oxygen species, protein carbonyl and advanced glycation end products in the mouse hippocampus [reactive oxygen species: F(5, 24) = 31.152, P < 0.001; protein carbonyl: F(5, 24) = 47.563, P < 0.001; advanced glycation end products: F(5, 24) = 29.139, P < 0.001 versus vehicle group], which induced endoplasmic reticulum stress [p-eIF2α (Ser51): F(5, 24) = 12.381, P < 0.01; p-PERK (Thr980): F(5, 24) = 4.117, P < 0.05 versus vehicle group]. Interestingly, troxerutin significantly reduced the levels of reactive oxygen species, protein carbonyl and advanced glycation end products induced by a high-cholesterol diet (reactive oxygen species: P < 0.001; protein carbonyl: P < 0.001; advanced glycation end products: P < 0.001 versus high-cholesterol diet group), which also alleviated endoplasmic reticulum stress [p-eIF2α (Ser51): P < 0.01; p-PERK (Thr980): P < 0.05 versus high-cholesterol diet group]. Moreover, the levels of reactive oxygen species, protein carbonyl and advanced glycation end products in the cholesterol/troxerutin group were restored to near-normal level (non-significant versus control group). The intra-cerebroventricular infusion of PI-103 (a specific PI3K inhibitor) did not inhibit this protective effect of troxerutin (non-significant versus vehicle group). No significant difference was found among the vehicle, troxerutin and PI-103 groups.
Troxerutin inhibits the activation of JNK1 and IKKβ/NF-κB induced by endoplasmic reticulum stress and attenuates insulin resistance in the hippocampus of mice fed a high-cholesterol diet

As shown in Fig. 2, a high-cholesterol diet significantly increased the expression of p-JNK1 (Thr183/Tyr185) [p-JNK1 (Thr183/Tyr185) immune fluorescence: F(5, 24) = 20.774, P < 0.001 versus vehicle group] and activated IKKβ through increasing the phosphorylation of IKKβ [p-IKKβ (Ser181): F(5, 24) = 16.705, P < 0.001 versus vehicle group], inducing the degradation of ΙκBα [ΙκBα: F(5, 24) = 5.302, P < 0.05 versus vehicle group] and stimulating nuclear translocation of NF-κB p65 [NF-κB p65Nuclear: F(5, 24) = 22.241, P < 0.001; NF-κB p65Nucleus: F(5, 24) = 6.597, P < 0.01 versus vehicle group] in the mouse hippocampus. To further evaluate whether activation of JNK1 and IKKβ/NF-κB had an effect on insulin signalling in the hippocampus of mice fed a high-cholesterol diet, we immunoprecipitated IRS1 and used western blot analysis to assess the phosphorylation status of IRS1 and IRS1 interactions with PI3K p110α (Fig. 3). Our results showed that activation of JNK1 and IKKβ/NF-κB in the hippocampus of these mice significantly increased serine phosphorylation of IRS1 and decreased IRS1-PI3K interactions.
IRS1 [p-IRS1 (Ser307): $F(5, 24) = 23.228, P < 0.001$ versus vehicle group] and reduced insulin signalling [PI3K p110α: $F(5, 24) = 23.351, P < 0.001$ versus vehicle group], which markedly decreased the phosphorylation of Akt [p-Akt (Ser473): $F(5, 24) = 19.768, P < 0.001$ versus vehicle group]. Similarly, a high-cholesterol diet significantly decreased the mean optical density of PI3K p85α [PI3K p85α: $F(5, 24) = 26.672, P < 0.001$ versus vehicle group]. In contrast, activation of JNK1 and IKKβ/NF-κB had no effect on total IRS1 expression [IRS1: $F(5, 24) = 0.078$, non-significant versus vehicle group] in the mouse hippocampus. Interestingly, oral administration of troxerutin to mice fed a high-cholesterol diet for 20 weeks could significantly inhibit these changes of protein expression ($P < 0.05$ versus high-cholesterol diet group). In addition, these levels of protein expression changes were restored to near-normal levels (non-significant versus vehicle group). The intra-cerebroventricular infusion of PI-103 to mice co-treated with troxerutin and cholesterol had no effect on the activation of JNK1 [p-JNK1 (Thr183/Tyr185): non-significant versus vehicle group] or IKKβ/NF-κB (non-significant versus vehicle group), phosphorylation of IRS1 (non-significant versus vehicle group) and expression of PI3K p85α (non-significant versus vehicle group), but significantly

Figure 2 Troxerutin inhibits the activation of JNK1 and IKKβ/NF-κB induced by endoplasmic reticulum stress in the hippocampus of high-cholesterol-fed mice ($n = 5$). All values are expressed as mean ± SD. (A) Representative immunoblot for p-JNK1 (Thr183/Tyr185), total-JNK1 (t-JNK1), p-IKKβ (Ser181), total-IKKβ (t-IKKβ), β-actin, and β-tubulin in all treated groups. (B) Relative density analysis of the p-JNK1, p-IKKβ, β-actin and β-tubulin protein bands. The relative density is expressed as the ratio (p-JNK1/t-JNK1; p-IKKβ/t-IKKβ; β-actin; β-tubulin). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus cholesterol group. (C) Hippocampal CA1 sections from all treated groups were stained with Cy5 and 4, 6-diamidino-2-phenylindole to visualize p-JNK1 (Thr183/Tyr185)-positive cells (red) and cell nuclei (blue), respectively. Scale bar = 25 μm. (D) Analysis of the mean optical density value of p-JNK1-positive cells in the hippocampal CA1 sections. ***$P < 0.001$ versus vehicle group; ###$P < 0.001$ versus cholesterol group.

Chol = cholesterol; DAPI = 4, 6-diamidino-2-phenylindole; IOD = integral optical density.
inhibited PI3K p110α expression (PI3K p110α: P < 0.01 versus vehicle group) and Akt phosphorylation [p-Akt (Ser473): P < 0.001 versus vehicle group]. There was no significant difference between the vehicle group and the troxerutin group.

Troxerutin prevents obesity and restores abnormal glucose, fatty acid and cholesterol metabolism induced by a high-cholesterol diet to near-normal levels

A substantial amount of evidence has demonstrated that obesity can induce excess lipid accumulation, abnormalities in intracellular energy fluxes and nutrient availability, might be a chronic stimulus for endoplasmic reticulum stress, insulin resistance and inflammation (Özcan et al., 2004; Boden, 2009). On the basis of these observations, we further examined body weight, the levels of blood insulin, glucose, leptin, free fatty acids, triglycerides and cholesterol, and glucose tolerance in each group (Fig. 4). Our data showed that after 11 weeks of a high-cholesterol diet, mice exhibited significant differences in body weight [F(5, 54) = 16.839, P < 0.001 versus vehicle group]. Moreover, feeding a high-cholesterol diet to mice for 19 weeks caused significantly increased levels of blood insulin, glucose, leptin, free fatty acids, triglycerides and cholesterol [insulin: F(5, 54) = 23.004, P < 0.001; glucose: F(5, 54) = 24.809, P < 0.001; leptin: F(5, 54) = 37.168, P < 0.001; free fatty acids: F(5, 54) = 6.168, P < 0.01; triglycerides: F(5, 54) = 21.056, P < 0.001; cholesterol: F(5, 54) = 92.827,
A glucose tolerance test was done for each group to investigate systemic insulin sensitivity. Exposure to a high-cholesterol diet resulted in significant glucose intolerance in mice. After 19 weeks of a high-cholesterol diet, mice showed significantly higher glucose levels on glucose challenge than the vehicle group. Hence, a high-cholesterol diet predisposed mice to peripheral insulin resistance and type 2 diabetes. However, oral administration of troxerutin to mice fed a high-cholesterol diet for 20 weeks markedly reversed the metabolic changes (\( P < 0.001 \) versus vehicle group). No significant difference was found among the vehicle, troxerutin and PI-103 groups.

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**Troxerutin increases the levels of phosphorylated cAMP response element-binding protein and c-fos in the hippocampus of mice fed a high-cholesterol diet**

Figure 5 shows the changes of memory-related proteins, including phosphorylated CREB and c-fos. Our results clearly indicated that
the levels of phosphorylated CREB and c-fos in the hippocampus of mice fed a high-cholesterol diet were markedly reduced [phosphorylated CREB: F(5, 24) = 14.92, P < 0.01; c-fos: F(5, 24) = 15.647, P < 0.01 versus vehicle group]. The administration of troxerutin significantly increased the levels of phosphorylated CREB and c-fos in the hippocampus of mice fed a high-cholesterol diet (phosphorylated CREB: P < 0.01; c-fos: P < 0.01 versus high-cholesterol diet group). The intra-cerebroventricular administration of PI-103 did not block the protective effect of troxerutin in the high-cholesterol/troxerutin group (non-significant versus high-cholesterol diet group). There was no significant difference between the vehicle and troxerutin groups.

**Troxerutin alleviates endoplasmic reticulum stress-induced apoptosis in the hippocampus of mice fed a high-cholesterol diet**

In this study, we investigated the effect of troxerutin on endoplasmic reticulum stress-induced apoptosis in the hippocampus of mice fed a high-cholesterol diet (Fig. 6). Western blot analysis showed that this diet significantly increased the activation of caspase-3 and caspase-12 [caspase-3: F(5, 24) = 4.857, P < 0.01; caspase-12: F(5, 24) = 5.793, P < 0.05 versus vehicle group] in the mouse hippocampus. In line with these results, the mean optical density of TUNEL-positive cells in the hippocampus of these mice was significantly increased [F(5, 24) = 9.658, P < 0.01 versus vehicle group]. The oral administration of troxerutin significantly decreased the activation of caspase-3 and caspase-12 (caspase-3: P < 0.05; caspase-12: P < 0.05 versus high-cholesterol diet group) and reduced the mean optical density of TUNEL-positive cells (P < 0.01 versus high-cholesterol diet group) in the hippocampus of mice fed a high-cholesterol diet. The intra-cerebroventricular administration of PI-103 did not block the protective effect of troxerutin in the high-cholesterol/troxerutin group (non-significant versus high-cholesterol/troxerutin group). There was no significant difference among the vehicle, troxerutin and PI-103 groups.

**Troxerutin improves cognitive deficits of mice fed a high-cholesterol diet**

**Step-through passive avoidance task**

In the acquisition trial, the initial latency did not differ among the six groups [F(5, 54) = 0.356, non-significant versus vehicle group] (Fig. 7). The step-through latency in the 24 h retention trial was significantly decreased in mice fed a high-cholesterol diet [F(5, 54) = 13.891, P < 0.001 versus vehicle group]. The analysis showed also that troxerutin significantly inhibited the amnestic effect of a high-cholesterol diet on latency time for the step-through trial (P < 0.01 versus high-cholesterol diet group), whereas the latency time in the high-cholesterol/troxerutin group was markedly decreased by PI-103 (P < 0.01 versus vehicle group). Similarly, the administration of PI-103 to vehicle group mice significantly shortened the step-through latency (P < 0.01 versus vehicle group). There was no significant difference in step-through latency among the high-cholesterol diet, high-cholesterol/PI-103/troxerutin and PI-103 groups and there was no significant difference in step-through latency between the vehicle group and the troxerutin group.

**Morris water maze task**

Statistical analysis for the escape latency in the Morris water maze test was done by two-way ANOVA for repeated measures with day and treatment as the sources of variation (Fig. 8). There was a significant difference in mean latency between training days [F(3, 216) = 78.694, P < 0.001 versus vehicle group] and between treatments [F(5, 216) = 11.305, P < 0.001 versus vehicle group], but there was no interaction between the factors day and treatment [F(15, 216) = 1.175, non-significant versus vehicle group].

The results also showed that mice fed a high-cholesterol diet had longer escape latency compared with vehicle mice (P < 0.001 versus vehicle group). A comparison between the high-cholesterol diet group and the high-cholesterol/troxerutin group showed that the administration of troxerutin could decrease the escape latency of mice fed a high-cholesterol diet (P < 0.01 versus high-cholesterol diet group) and there was no significant difference in spatial learning or memory ability between the vehicle group and the high-cholesterol/troxerutin group. However, PI-103 blocked the protective effect of troxerutin in the high-cholesterol/troxerutin group (P < 0.01 versus high-cholesterol/troxerutin group). PI-103 also significantly decreased escape latency of vehicle mice. There was no significant difference in escape latency among the high-cholesterol diet, high-cholesterol/PI-103/troxerutin and PI-103 groups. There was also no significant difference in escape latency between the vehicle group and the troxerutin group. Note that all of the mice had the same level of performance at the start of the experiment (no significant individual effect was observed for the first four trials of Day 1).

On the fifth test day, the platform was removed and the probe test was done. The vehicle, high cholesterol/troxerutin and troxerutin groups spent more time swimming in the target quadrant (where the platform was located) [F(5, 54) = 28.104, non-significant versus vehicle group] than the high-cholesterol diet group (P < 0.001 versus vehicle group). The intra-cerebroventricular administration of PI-103 to mice fed a high-cholesterol diet treated orally with troxerutin blocked the neuroprotective effect of troxerutin (P > 0.001 versus high cholesterol/troxerutin diet group). Similar results were obtained for the mean number of times the animals crossed the place where the platform was located during training. Mice fed a high cholesterol crossed over the platform less frequently than vehicle mice [F(5, 54) = 22.080, P < 0.001 versus vehicle group]. No significant difference between the vehicle group and the high-cholesterol/troxerutin group was found as well as the difference between the vehicle group and the troxerutin group. PI-103 blocked the neuroprotective effect of troxerutin in the high-cholesterol/troxerutin group (P < 0.001 versus high-cholesterol/troxerutin group).
Discussion

Clinical and epidemiological data show that obesity and obesity-related diseases have increased exponentially all over the world, which might result from an increase in dietary cholesterol and/or saturated fat, caloric input and a decrease in physical activity (Mattson, 2000). A substantial amount of evidence indicates that obesity can contribute to the development of type-2 diabetes and ultimately result in cognitive function deficits that might be caused by neuronal insulin resistance, brain inflammation and...
impaired neural signalling pathways (Elias et al., 2003; Özcan et al., 2004; Stranahan et al., 2008b; Toro et al., 2009). Evidence from earlier studies showed that a high-cholesterol diet-induced oxidative stress and inflammatory response and ultimately results in neurodegeneration and cognitive deficits (Sparks and Schreurs, 2003; Thirumangalakudi et al., 2008; Lu et al., 2009). Recent findings indicate that a high-cholesterol diet also induces insulin resistance (Hahn-Obercyger et al., 2009; Taghibiglou et al., 2009), which is considered to be an underlying mechanism of neurotoxicity. Advanced glycation end products are markers of carbonyl stress and formed in excess during ageing and diabetes mellitus (Wautier and Guillausseau, 2002; Srikanth et al., 2009). There is evidence that advanced glycation end products and oxidative stress induced by a high-cholesterol diet can both trigger endoplasmic reticulum stress, which impairs insulin action through JNK and IKKβ/NF-κB-mediated phosphorylation of IRS1 (Sparks and Schreurs, 2003; Özcan et al., 2004; Tokita et al., 2005; Chen et al., 2008; Sato et al., 2010). In the present study, our results demonstrated that a high-cholesterol diet significantly increased advanced glycation end products, reactive oxygen species and protein carbonyl levels in the mouse hippocampus, which promoted the phosphorylation of PERK and eIF2α (endoplasmic reticulum stress markers). Subsequently, endoplasmic reticulum stress impaired insulin signalling including increased serine phosphorylation of IRS1 and suppressed insulin-stimulated PI3K and PKB/Akt activities by triggering JNK activity and inducing IKKβ/NF-κB activation. Chronic inflammation mediated by IKKβ/NF-κB activation is a hallmark of obesity, which in turn might be a

![Figure 6](https://academic.oup.com/brain/article-abstract/134/3/783/447400/10315316)

**Figure 6** Troxerutin alleviates endoplasmic reticulum stress-induced apoptosis in the hippocampus of high-cholesterol-fed mice (n = 5). All values are expressed as mean ± SD. (A) Representative immunoblot for caspase-12, cleaved caspase-3 and β-actin in all treated groups. (B) Relative density analysis of the caspase-12 and cleaved caspase-3 protein bands. The relative density is expressed as the ratio (caspase-12/β-actin; cleaved caspase-3/β-actin). *P < 0.05, **P < 0.01 versus vehicle group; #P < 0.05, ##P < 0.01 versus cholesterol group. (C) In situ detection of fragmented DNA (TUNEL assay) in the hippocampal CA1 of all treated groups. The hippocampal CA1 were processed for TUNEL and photographed using a fluorescence microscope with either a fluorescein isothiocyanate (FITC) filter alone (left), or a propidium iodide (PI) filter alone (middle). The merged images shows that apoptotic cells appear yellow and non-apoptosis cells appear red (right). Scale bar = 25 μm. (D) Analysis of the mean optical density value of TUNEL-positive cells in the hippocampal CA1 sections. **P < 0.01 versus vehicle group; **P < 0.01 versus cholesterol group. Chol = cholesterol; IOD = integral optical density.
chronic stimulus for endoplasmic reticulum stress in peripheral tissues (Özcan et al., 2004; Zhang et al., 2008; Nakamura et al., 2010). So we further assessed body weight and the levels of blood insulin, glucose, leptin, free fatty acids, triglycerides and cholesterol and glucose tolerance in each group. Consistent with earlier observations (Özcan et al., 2004; Zhang et al., 2008; Nakamura et al., 2010), a high-cholesterol diet significantly induced abnormal changes of these parameters, peripheral insulin resistance and type 2 diabetes. The PI3K/Akt pathway is an important regulator of several important cellular processes, including apoptosis, survival, proliferation, and metabolism. Our earlier report demonstrated that the PI3K/Akt pathway is also involved in the learning and memory processes (Lu et al., 2010a). When the PI3K/Akt pathway was inhibited by endoplasmic reticulum stress in the hippocampus of mice fed a high-cholesterol diet, the expression of memory-related proteins in the downstream part of the PI3K/Akt pathway, such as phosphorylated CREB and c-fos, was significantly downregulated and ultimately led to behaviour deficits, which agrees with the recent experimental and clinical view that impaired insulin signalling in the brain of obese diabetic mice induces cognitive decline (Zhu et al., 2005). It is possible that endoplasmic reticulum stress induces apoptosis through JNK upregulation and caspase-12 activation, which has been linked to neurodegenerative diseases (Zhao and Ackerman, 2006). Our present experimental data confirmed that endoplasmic reticulum stress-induced apoptosis in the hippocampus of mice fed a high-cholesterol diet was mediated by JNK upregulation and caspase-12 activation that resulted in a significant increase in the expression levels of cleaved caspase-3 and the mean optical density of TUNEL-positive cells. These changes might not be the primary cause of neuron death; however, it might modify the progression and severity of high-cholesterol-induced cognitive deficits.

Troxerutin, a rutin derivative, is known to possess antioxidant and anti-inflammatory effects (Blasig et al., 1987, 1988; Fan et al., 2009; Zhang et al., 2009). Our recent studies demonstrated that troxerutin exerts neuroprotective effects through decreasing advanced glycation end products, reactive oxygen species and protein carbonyl levels and enhancing PI3K/Akt activation

Figure 7 Step-through passive avoidance task (n = 10). All values are expressed as mean ± SD. ***P < 0.001 versus vehicle group; ****P < 0.001 versus cholesterol (Chol) group.

Figure 8 Morris water maze task (n = 10). All values are expressed as mean ± SD. (A) Mean latency in the hidden platform test. (B) Comparison of the time spent in target quadrant (where the platform was located during hidden platform training) and the number of crossings over the exact location of the former platform on Day 5. ***P < 0.001 versus vehicle group; ****P < 0.001 versus cholesterol (Chol) group.
In conclusion, troxerutin reverses high-cholesterol-induced cognitive deficits through activating the PI3K/Akt/CREB pathway and inhibiting the endoplasmic reticulum stress pathway in the mouse hippocampus, and could be recommended as a possible candidate for the prevention and therapy of cognitive deficits in type 2 diabetes mellitus and Alzheimer’s disease. A diagram of the neuroprotective effects of troxerutin against high-cholesterol-induced cognitive deficits is shown in Fig. 9.

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