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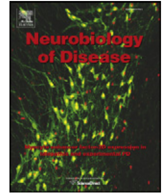

Lutein enhances survival and reduces neuronal damage in a mouse model of ischemic stroke

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ABSTRACT

Introduction: Stroke is one of the leading causes of death worldwide. Protective agents that could diminish the injuries induced by cerebral ischemia/reperfusion (I/R) are crucial to alleviate the detrimental outcome of stroke. The aim of this study is to investigate the protective roles of lutein in cerebral I/R injury.
Methods: Two-hour cerebral ischemia was induced by unilateral middle cerebral artery occlusion (MCAo) in mice. Either lutein (0.2 mg/kg) or vehicle was given to mice intraperitoneally 1 h after MCAo and 1 h after reperfusion. Neurological deficits were evaluated at 22 h after reperfusion while survival rate was assessed daily until 7 days after reperfusion. Brains were cut into 2 mm-thick coronal slices and stained with 2% 2,3,5-triphenyltetrazolium chloride to determine the infarct size after MCAo. Paraffin-embedded brain sections were prepared for TUNEL assay and immunohistochemistry. Protein lysate was collected for Western blotting experiments.
Results: Higher survival rate, better neurological scores, smaller infarct area and smaller infarct volume were noted in the lutein-treated group. Immunohistochemistry data showed a decrease of immunoreactivity of nitrotyrosine, poly(ADP-ribose) and NFκB in the lutein-treated brains. Western blotting data showed decreased levels of Cox-2, pERK, and pIκB, but increased levels of Bcl-2, heat shock protein 70 and pAkt in the lutein-treated brains.
Conclusions: Post-treatment of lutein protected the brain from I/R injury, probably by its anti-apoptotic, anti-oxidative and anti-inflammatory properties. These suggest that lutein could diminish the deleterious outcomes of cerebral I/R and may be used as a potential treatment for stroke patients.
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Introduction

Stroke is a major cause of death and disability in the world (Donnan et al., 2008). Nearly 85% of strokes are acute ischemic stroke which results from a sudden loss of blood supply to the cerebral regions and consequently leads to neuronal death, and disability (Lakhan et al., 2009). Treatment of ischemic stroke is important to alleviate the subsequent outcome of stroke.

Our brain is susceptible to damages caused by oxidative stress in cerebral ischemia/reperfusion (I/R) injury due to its high lipid content (Mehta et al., 2007). Excess generation of free radicals and reactive oxygen species (ROS) overwhelm the endogenous anti-oxidative mechanisms during I/R by decreasing the scavenging capacity of anti-oxidative enzymes and depleting non-enzymatic anti-oxidants, resulting in oxidative injury to tissues (Mehta et al., 2007). Oxidative stress induces deleterious damages via the activation of signaling pathways such as PI3K/Akt, mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NFκB)(Namura et al., 2001; Song et al., 2008; Song et al., 2010).

Post-ischemic inflammation also plays an important role in cerebral I/R damage (Lakhan et al., 2009). NFκB is a transcription factor that is involved in the regulation of genes and cellular response during post-ischemic inflammation. It is controlled by the upstream kinases, the IκB kinases (IKKs) and its inhibitory unit IκB. Therefore, inhibition of the NFκB-related signaling pathway may interrupt the consequent injury due to inflammation (Ridder and Schwaninger, 2009).

Lutein, a xanthophyll, is rich in green leafy vegetables, such as spinach and kale (Sommerburg et al., 1998). It is a potent anti-oxidant and is characterized by having a hydroxyl group attached to each end of the molecule. This unique structure makes it more hydrophilic and reacts more strongly with singlet oxygen than other carotenoids (Ribaya-Mercado and Blumberg, 2004). Previously, we have shown that lutein post-treatment protects retinal neurons from hypoxia and I/R injury by decreasing oxidative stress and apoptosis (Li and Lo, 2010; Li et al., 2009). As lutein has been proved as a safe daily supplement for ocular health, it is crucial to investigate its neuroprotective effects in cerebral

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I/R injury, which may be beneficial in minimizing the unfavorable outcomes of stroke. In the present study, we focused on investigating the anti-apoptotic, anti-oxidative and anti-inflammatory effects of lutein, including the underlying signaling pathways involved in cerebral I/R injury.

Materials and methods

Animals

The use of animals in this study was conducted according to the requirements of the Cap. 340 Animals (Control of Experiments) Ordinance and Regulations, and all relevant legislation and Codes of Practice in Hong Kong. All the experimental and animal handling procedures were approved by the Faculty Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong (CULATR #1648-08).

C57BL/6N male mice (10–12 weeks old) were used and kept in a temperature-controlled room with 12-hour light/12-hour dark cycle in the Laboratory Animal Unit of The University of Hong Kong. All the experimental and animal handling procedures were approved by the Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong.

Animals were divided into three groups: Group A was used for studies on survival rate, histological and immunohistochemical analyses at 7 days after reperfusion; Group B was used for histological and immunohistochemical studies at 22 h after reperfusion and Group C was used for Western blotting experiment at 22 h after reperfusion.

Cerebral I/R

Ischemic stroke was induced using middle cerebral artery occlusion (MCAo) method which has been described previously (Li and Lo, 2010; Li et al., 2009; Lo et al., 2005; Lo et al., 2007). Briefly, mice were anesthetized (2% halothane in 70% N_2O/30% O_2 for induction and 1% halothane in 70% N_2O/30% O_2 for maintenance) and MCAo was induced by inserting an 8/0 nylon monofilament coated with vinyl polysiloxane impression material (3M Dental Products, St. Paul, MN) through the right external carotid artery into the right internal carotid artery to occlude the right middle cerebral artery at its origin. To ensure successful blockade of the artery, an optic fiber was glued to the skull (2 mm posterior and 6 mm lateral to bregma) and connected to a laser Doppler flowmeter (Perimed, Järfälla, Sweden) for monitoring the relative regional cerebral blood flow (rCBF) in the core territory of the right middle cerebral artery. MCAo was maintained for 2 h after which the filament was pulled out to allow reperfusion for 7 days (Group A) or for 22 h (Group B & Group C).

Treatment

Lutein (0.2 mg/kg; Sigma-Aldrich Co, St. Louis, MO) or vehicle (10% dimethyl sulfoxide, DMSO) at 4 ml/kg (i.e. 0.1 ml/25 g) was administered by intraperitoneal injection 1 h after MCAo and 1 h after reperfusion in all animals. This dosage of lutein was chosen following our previous publication (Li et al., 2009). Daily injection of either lutein or vehicle was also given to animals in Group A for 7 days.

Neurological deficits, infarct area, infarct volume and hemispheric swelling

Animals in Groups A, B and C were evaluated for neurological deficits at 22 h after reperfusion. Briefly, mice were scored as follows: 0, no observable neurological deficits (normal); 1, failure to extend opposite forepaw (mild); 2, circling to the contralateral side (moderate) and 3, loss of walking and righting reflex (severe). After the designated experimental periods, brains from Group A and Group B animals were cut into 5 coronal slices of 2 mm thickness, stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C in dark for 10 min to demarcate infarct area. The posterior surface of each brain slice was photographed and analyzed using a digital image analysis system (SigmaScan Pro, SPSS, Chicago, IL) for estimation of infarct area and infarct volume using the indirect method (Lo et al., 2005; Lo et al., 2007). Hemispheric swelling was determined as 100% × (ipsilateral volume − contralateral volume) / contralateral volume (Huang et al., 1994).

Tissue processing

Brain slices of Group A and Group B animals were fixed, dehydrated and embedded in paraffin. Seven-μm cross-sections were cut using a microtome (Microm HM 315R, Heidelberg, Germany).

Terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL)

Apoptosis in the brain sections of Group A and Group B animals was examined by TUNEL assay (DeadEnd Fluorometric TUNEL system, Promega, Madison, WI) (Li et al., 2009; Li et al., 2011). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) after the reaction to verify that the TUNEL staining is localized in the nucleus. Five cortical regions (area 0.07 mm^2) at the interaural line of approximately 4.18 mm (Franklin and Paxinos, 1997) in the ipsilateral ischemic side were selected. The number of TUNEL-positive cells that were colocalized with DAPI staining was counted.

Immunohistochemistry (IHC)

After antigen retrieval and blocking, sections were incubated with primary antibodies: phospho-NFκB (pNFκB; 1:1000; Cell Signaling Technology, CST, Beverly, MA), nitrotyrosine (NT; 1:200; Upstate Biotechnology, Lake Placid, NY) and poly(ADP-ribose) (PAR; 1:200; Alexis, Lausen, Switzerland). Signals were detected by Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine tetrahydrochloride (Zymed, South San Francisco, CA). The sections were then washed and cover-slipped for examination. Photomicrographs were captured with a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) equipped with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Semi-quantitative analysis was performed to assess the immunoreactivity in a double-blinded approach (Li et al., 2011; Yeung et al., 2010). IHC scores were given according to the level of immunoreactivity. Score 1 represented the weakest immunoreactivity while score 5 indicated the highest immunoreactivity.

Western blotting

Protein lysate of brains from Group C animals was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk, membranes were incubated with various primary antibodies: actin (1:1000; Chemicon, Temecula, CA), Akt (1:1000; CST), Bcl-2 (1:1000; CST), Cox-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), ERK (1:1000; CST), Hsp-70 (1:1000; Stressgen Biotechnology, Victoria, BC, Canada), IκB (1:1000; CST), phospho-Akt (pAkt; 1:1000; CST), phospho-ERK (pERK; 1:1000; CST), and phospho-IκB (pIκB; 1:1000: CST) for overnight at 4 °C. After secondary antibody incubation, signals were detected by ECL (Amersham Pharmacia Biotech, Arlington Heights, IL). The signals on the films were scanned and quantified using Image J software (National Institute of Mental Health, Bethesda, MD).

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