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Neuroprotective Effects of Xanthohumol, a Prenylated Flavonoid from Hops (*Humulus lupulus*), in Ischemic Stroke of Rats

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ABSTRACT: Xanthohumol is the principal prenylated flavonoid in hops (*Humulus lupulus* L.), an ingredient of beer. Xanthohumol was found to be a potent chemopreventive agent; however, no data are available concerning its neuroprotective effects. In the present study, the neuroprotective activity and mechanisms of xanthohumol in rats with middle cerebral artery occlusion (MCAO)-induced cerebral ischemia were examined. Treatment with xanthohumol (0.2 and 0.4 mg/kg; intraperitoneally) 10 min before MCAO dose-dependently attenuated focal cerebral ischemia and improved neurobehavioral deficits in cerebral ischemic rats. Xanthohumol treatment produced a marked reduction in infarct size compared to that in control rats. MCAO-induced focal cerebral ischemia was associated with increases in hypoxia-inducible factor (HIF)-1α, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and active caspase-3 protein expressions in ischemic regions. These expressions were obviously inhibited by treatment with xanthohumol. In addition, xanthohumol (3−70 μM) concentration-dependently inhibited platelet aggregation stimulated by collagen (1 μg/mL) in human platelet-rich plasma. An electron spin resonance (ESR) method was used to examine the scavenging activity of xanthohumol on free radicals which had formed. Xanthohumol (1.5 and 3 μM) markedly reduced the ESR signal intensity of hydroxyl radical ($OH^{\bullet}$) formation in the H_2O_2/NaOH/DMSO system. In conclusion, this study demonstrates for the first time that in addition to its originally being considered an agent preventing tumor growth, xanthohumol possesses potent neuroprotective activity. This activity is mediated, at least in part, by inhibition of inflammatory responses (i.e., HIF-1α, iNOS expression, and free radical formation), apoptosis (i.e., TNF-α, active caspase-3), and platelet activation, resulting in a reduction of infarct volume and improvement in neurobehavior in rats with cerebral ischemia. Therefore, this novel role of xanthohumol may represent high therapeutic potential for treatment or prevention of ischemia-reperfusion injury-related disorders.

KEYWORDS: *HIF-1α, MCAO, hydroxyl radical, xanthohumol, TNF-α, platelet activation*

INTRODUCTION

Ischemic hypoxic brain injury often causes irreversible brain damage. The cascade of events leading to neuronal injury and death in ischemia includes the release of cytokines, excitatory amino acids, nitric oxide (NO),[1,2] and free radicals,[1,3] as well as damage to mitochondrial respiratory enzymes, induction of programmed cell death, and microglial activation.[1−3] Reperfusion of ischemic areas can exacerbate ischemic brain damage through the generation of reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), and NO.[4] Leukocytes are a potential source of ROS when activated during inflammatory responses.[5] When a tissue suffers from ischemia and reperfusion, proinflammatory cytokines produced by inflammatory cells can trigger adhesion and migration of circulating leukocytes to endothelial cells and ROS generation which enhances ischemic injury.[6] On the other hand, the participation of activated platelets was observed in brain microvessels of the ischemic microvascular bed after experimental middle cerebral artery occlusion (MCAO).[7] Microvascular thrombi continue to accumulate even after recanalization of the MCAO, contributing to postischemic hypoperfusion and ongoing neuronal damage.[8] Thus, platelet aggregation plays a crucial role in MCAO-induced cerebral damage.

Hops (*Humulus lupulus* L.) have been used for more than 500 years to preserve beer, and they impart a characteristic aroma and flavor.[9] In traditional Chinese medicine, hops are used to treat insomnia, restlessness, dyspepsia, and a lack of appetite. Alcoholic extracts of hops have been clinically used in China to treat leprosy, pulmonary tuberculosis, acute bacterial dysentery, silicosis, and asbestosis with positive outcomes.[9] Xanthohumol (2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone) is the principal prenylated chalcone (open C-ring flavonoids) in the hop plant (Figure 1). Recently, xanthohumol has attracted considerable interest because of its biological activities, including anticancer, antiangiogenesis, anti-inflammation, and antioxidation.[10] Xanthohumol (1−50 μM) suppressed tumor growth by inhibiting cell proliferation and inducing apoptosis in various carcinoma cells.[11−13] It also exhibited antiangiogenic

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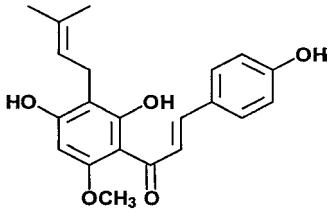

Figure 1. Chemical structure of xanthohumol.

activity through inhibiting nuclear factor (NF)-κB and Akt activation in vascular endothelial cells.[14] Furthermore, xanthohumol was reported to regulate the function and survival of immune cells by inhibiting the production of two important cytokines, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α, in lipopolysaccharide (LPS)-stimulated macrophages.[15] Xanthohumol also prevented hepatic inflammation and fibrosis in vivo and promoted dendritic cell apoptosis.[9,16] Furthermore, xanthohumol was able to scavenge ROS in tissue plasminogen activator (TPA)-stimulated differentiated HL-60 cells.[10]

Recently, Lee et al.[17] reported that xanthohumol exerts anti-inflammatory activity through the transcription factor, NF-E2-related factor (NRF) 2 antioxidant responsive element (ARE) signaling in microglial cells, and suggested that it may be an attractive candidate to regulate inflammatory responses in the brain. However, no data are available concerning the neuroprotective effects of xanthohumol so far, besides its well-known antitumor properties. Considering the pivotal roles of platelet aggregation, free radicals, and inflammatory responses in ischemia/reperfusion-induced brain injury, the present study was designed to examine the potential neuroprotective effects and its mechanisms of xanthohumol's using an MCAO-reperfusion model in rats.

MATERIALS AND METHODS

Materials. Xanthohumol (≥96%), 2,3,5-triphenyltetrazolium (TTC), collagen (type I), Cremophor EL, 5,5-dimethyl-1 pyrroline *N*-oxide (DMPO), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Xanthohumol was dissolved in a solvent (cremophor:ethanol:normal saline at 1:1:4) for the in vivo study, and was dissolved in 0.5% DMSO for the in vitro studies.

MCAO-Induced Transient Focal Cerebral Ischemia in Rats. Male Wistar rats (250−300 g) were used in this study. All animal experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). Before undergoing the experimental procedures, all animals were confirmed to be clinically normal, were free of apparent infection or inflammation, and showed no neurological deficits, as evaluated by a spontaneous rotational test.

Animals were anesthetized with a mixture of 75% air and 25% O_2 gases containing 3% isoflurane. The rectal temperature was maintained at 37 ± 0.5 °C. The right middle cerebral artery (MCA) was occluded as described in our previous report.[18] Briefly, the right common carotid artery was exposed, and a 4-0 monofilament nylon thread (25 mm) coated with silicon was inserted from the external to the internal carotid artery until the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awake from the anesthesia. During another brief period of anesthesia, the filament was gently removed after 1 h of MCAO. An observer blinded to the identity of the groups assessed the neurological deficits at 1 and 24 h after reperfusion (before euthanization) by a forelimb akinesia (also called the postural tail-hang) test, whereas a spontaneous rotational test was used as a criterion for evaluating the ischemic insult.[19] Animals not showing behavioral deficits at the above time points after reperfusion were excluded from the study. On the other hand, reperfusion was also ensured by an improvement in the ipsilateral local blood flow to at least 60% of the baseline following an initial sharp decrease to about 50%−60% of the baseline caused by MCAO as determined using a continuous laser Doppler flow meter (LDF; Oxford Array, Oxford Optronix, Oxford, U.K.) with a standard needle probe (pp-051).

Rats were euthanized by decapitation after 24 h of reperfusion. The brains were cut into 2 mm coronal slices starting 1 mm from the frontal pole. Each stained brain slice (stained with 2% TTC) was drawn using a computerized image analyzer (Image-Pro plus). The calculated infarct areas were compiled to obtain the infarct volume (mm^3) per brain. Infarct volumes were expressed as a percentage of the contralateral hemisphere volume using the formula (area of the intact contralateral [left] hemisphere) − (area of the intact region of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere.[18]

All animals were divided into five groups: (1) a sham-operated group; (2) a normal saline (NS)-treated group, (3) a solvent (Cremophor:ethanol:normal saline at 1:1:4)-treated group; and groups treated with a single dose of (4) 0.2 or (5) 0.4 mg/kg, ip of xanthohumol. Rats received the isovolumetric normal saline, solvent, or xanthohumol (0.2 or 0.4 mg/kg) 10 min before MCAO was performed.

Neurobehavioral Test. Sensorimotor integrity was evaluated to assess the neurobehavior at 1 and 24 h after MCAO in rats.[19] Five categories of motor neurological findings were scored: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to a lateral push; 3, forelimb flexion, decreased resistance to a lateral push, and unilateral circling; 4, forelimb flexion and difficult or impossible ambulation.

Determination of Protein Expressions by an Immunoblotting Assay in MCAO-Insulted Brains. Expressions of hypoxia-inducible factor (HIF)-1α, TNF-α, inducible nitric oxide synthase (iNOS), and activated caspase-3 in the brain at 24 h after MCAO-reperfusion injury were analyzed by Western blotting as described by Rodrigo et al.[20] with minor modifications. MCAO-insulted and sham-operated rats were anesthetized with chloral hydrate (400 mg/kg, ip), and then the apex of the heart was penetrated with a perfusion cannula inserted through the left ventricle into the ascending aorta. Perfusion with ice-cold phosphate-buffered saline (PBS) was performed, and an incision was made in the right atrium for venous drainage. Brains were removed and immediately sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third part of the right hemisphere was separately collected, snap-frozen in liquid nitrogen, and stored at −70 °C. The frozen tissues were placed in homogenate buffer and homogenized, then sonicated for 10 s three times at 4 °C. The sonicates were subjected to centrifugation (10000*g*).

The supernatant (50 μg of protein) was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes (0.45 μm; Hybond-P; Amersham). After incubation in blocking buffer and being washed three times with TBST buffer (10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5), blots were treated with an anti-HIF-1α polyclonal antibody (pAb, 1:1000; R&D, Minneapolis, MN), an anti-TNF-α pAb (1:1000; Cell Signaling, Beverly, MA), an anti-iNOS monoclonal antibody (mAb; 1:3000, BD Biosciences, San Jose, CA), and an antiactive caspase-3 pAb (1:250; Biovision, Mountain View, CA), or an anti-α-tubulin mAb (1:2000; Santa Cruz Biotech, Santa Cruz, CA) in TBST buffer overnight. Blots were subsequently washed with TBST and incubated with a secondary horseradish peroxidase-conjugated goat anti-mouse mAb or donkey anti-rabbit immunoglobulin G (IgG) (Amersham) for 1 h. Blots were then washed, and the immunoreactive protein was detected using film exposed to enhanced chemiluminescence detection reagents (ECLplus system; Amersham). A bar graph was drawn to depict ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-1D vers. 99 image software).

Platelet Aggregation. Human platelet-rich plasma (PRP) was prepared as previously described.[21] This study was approved by the Institutional Review Board of Taipei Medical University and conformed to the principles outlined in the *Helsinki Declaration*, and all human volunteers provided informed consent. In brief, blood was collected from healthy human volunteers who had taken no medication during the preceding 2 weeks, and was mixed with acid/citrate/glucose. After centrifugation, the supernatant (PRP) was obtained for the aggregation study.

A turbidimetric method was used to measure platelet aggregation,[21] with a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). PRP was preincubated with various concentrations of xanthohumol (3, 35, and 70 μM) or an isovolumetric solvent control (0.5% DMSO) for 3 min before the addition of collagen. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed in light-transmission units.

Electron Spin Resonance (ESR) Spectrometry. ESR spectra were recorded on a Bruker EMX ESR spectrometer in the H_2O_2/NaOH/DMSO system as described previously.[22] Briefly, 100 μL of DMSO and the same volumes of 25 mM NaOH and xanthohumol (1.5 and 3 μM) were mixed in a test tube, followed by the addition of 10 μL of DMPO and 100 μL of 30% H_2O_2. The reaction mixture was aspirated into a quartz flat cell and set in the ESR apparatus; scanning was begun 1 min after all reagents were mixed. The rate of free radical-scavenging activity was defined by the following equation: inhibition rate = 1 − {[signal height (xanthohumol)]/[signal height (solvent control)]}.[22]

Statistical Analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Student's unpaired *t*-test was used to determine significant differences in the study of MCAO-induced cerebral ischemia. Other experiments were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among group means, then each group was compared using the Newman−Keuls method. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of Xanthohumol on MCAO-Induced Focal Cerebral Ischemia in Rats. All animals in this study showed similar physiological values (i.e., rectal temperature and mean arterial blood pressure) before, during, and after MCAO among groups (data not shown). Neither abnormal behavior, depression of respiration, nor hypothermia was observed in the solvent- or xanthohumol-treated groups. The cerebral infarction was examined using 2 mm thick slices of the cerebrum 24 h after MCAO reperfusion in rats through TTC staining. Figure 2A shows typical photographs of coronal sections of sham-operated, normal saline (NS)-treated, solvent (Cremophor:ethanol:normal saline at 1:1:4)-treated, and xanthohumol-treated groups (0.2 and 0.4 mg/kg) prior to the ischemic insult. Administration of xanthohumol at 0.2 and 0.4 mg/kg showed dose-dependent reductions in infarct volume (white area) compared to the solvent-treated group (solvent, 50.6 ± 2.2% vs 0.2 mg/kg, 34.9 ± 2.4%; 0.4 mg/kg, 19.8 ± 1.7%, n = 7) (Figure 2B). On the other hand, treatment with the solvent did not significantly influence the infarct size compared to that of the normal saline (NS)-treated group (Figure 2A,B).

Figure 3A gives statistical results of the infarct areas of solvent- and xanthohumol (0.4 mg/kg)-treated groups at various distances from the frontal pole. The infarct area was largest between the second and fourth sections in both groups. Treatment with xanthohumol (0.4 mg/kg) markedly reduced the infarct area in all regions (Figure 3A). On the other hand, the neurobehavioral function of xanthohumol (0.2 and 0.4 mg/kg)-treated rats had more obviously improved at 24 h after

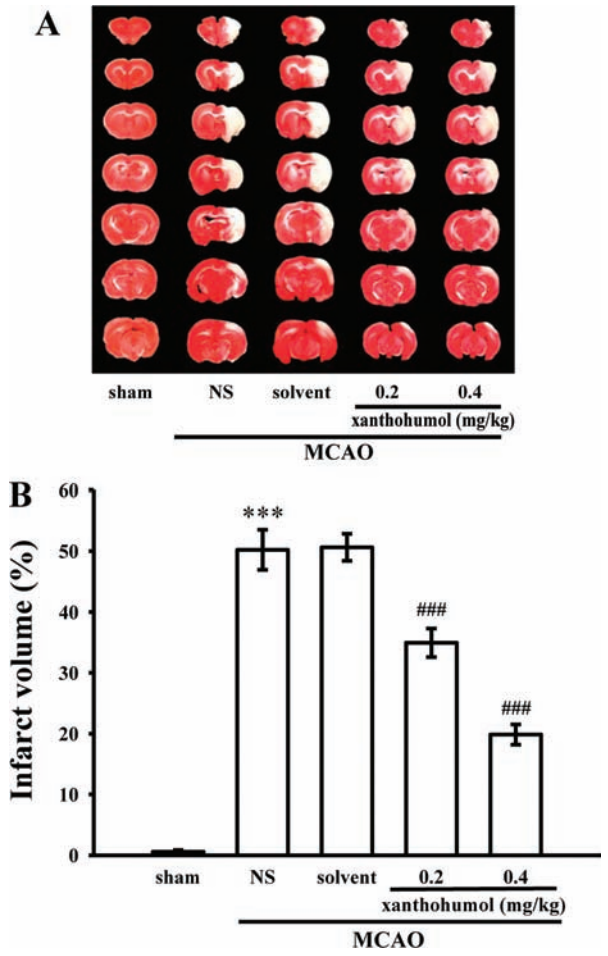

Figure 2. Effects of xanthohumol on middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia in rats. (A) Coronal sections of 2,3,5-triphenyltetrazolium (TTC)-stained brains and (B) dose−response effect of xanthohumol at 24 h after MACO-reperfusion in rats. Cerebral infarction in sham-operated (sham) or MACO-reperfusion rats from a representative animal that received normal saline (NS), solvent (Cremophor:ethanol:normal saline at 1:1:4), or xanthohumol (0.2 and 0.4 mg/kg) intraperitoneally. (A) TTC-stained brains and (B) infarct volumes were calculated as described in Materials and Methods, and data are presented as the percentage of infarct volume of each animal in the group (means ± SEM; n = 7). ***P < 0.001 compared to the sham-operated group; ###P < 0.001 compared to the solvent-treated group.

MCAO than that of the solvent-treated group (solvent-treated group, 3.6 ± 0.2; 0.2 mg/kg xanthohumol, 2.4 ± 0.2; 0.4 mg/kg xanthohumol, 1.6 ± 0.2; n = 7) (Figure 3B).

Effects of Xanthohumol on TNF-α, Active Caspase-3, HIF-1α, and iNOS Protein Expressions in Ischemic Cerebral Tissues. Results of Western blotting of MCAO-insulted cerebral tissues are shown in Figures 4 and 5. As shown in Figure 4A, negative immunostaining was obtained for TNF-α in the sham-operated group. At 24 h after MCAO-reperfusion, strong staining of TNF-α was observed in ischemic cerebral tissues (normal saline-treated) compared to levels obtained in the corresponding area of the sham-operated group. Treatment with the solvent did not significantly influence TNF-α expression compared to the normal saline-treated group (Figure 4A). Xanthohumol (0.2 and 0.4 mg/kg) obviously abolished the elevated TNF-α expression (Figure 4A). In addition, transient MCAO resulted in a significant increase in

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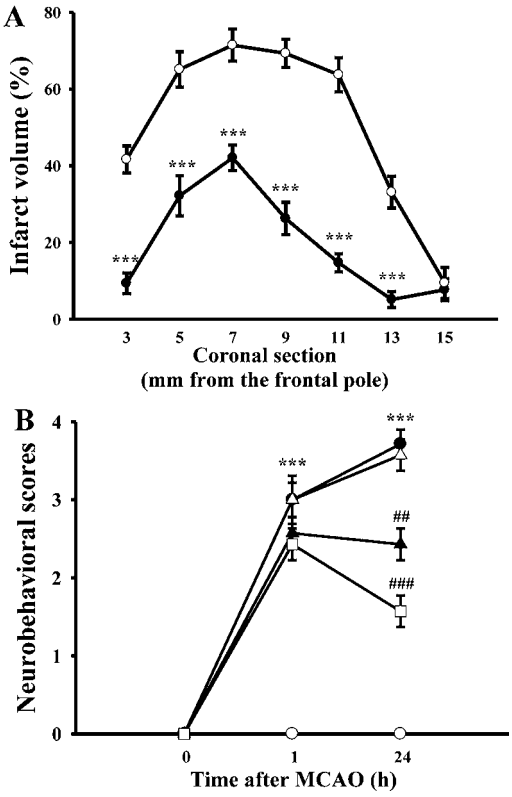

Figure 3. Reduction of the infarct volume and improvement of neurobehavior by xanthohumol in middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia in rats. (A) Infarct area at various distances from the frontal pole (○, solvent-treated group; ●, 0.4 mg/kg xanthohumol-treated group) and (B) neurobehavioral scores (○, sham-operated group; ●, normal saline-treated group; Δ, solvent-treated group; ▲, 0.2 mg/kg xanthohumol-treated group; □, 0.4 mg/kg xanthohumol-treated group) in rats at 1 and 24 h after MACO-reperfusion as described in Materials and Methods. Each point represents the mean ± SEM (n = 7). (A) $^{***}P < 0.001$ compared to the solvent-treated group and (B) $^{***}P < 0.001$ compared to the sham-operated group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to the solvent-treated group.

the expression of active caspase-3 (17 kDa) in the injured hemisphere (normal saline-treated) compared to levels obtained in the corresponding area of the sham-operated group (Figure 4B). Xanthohumol (0.2 and 0.4 mg/kg) treatment also markedly reduced active caspase-3 expression (Figure 4B).

HIF-1α, detected as a major band of approximately 120 kDa at 24 h after MCAO-reperfusion injury (normal saline-treated), was more pronounced than that of levels obtained in the corresponding area of the sham-operated group (Figure 5A). Xanthohumol (0.4 mg/kg) treatment markedly ($P < 0.001$) suppressed the expression of HIF-1α in ischemic cerebral tissues compared to levels obtained in the corresponding area of the solvent-treated group (Figure 5A). In Figure 5B, the iNOS band, detected as a major band of approximately 135 kDa, showed significant increases in ischemic cerebral tissues (normal saline-treated) 24 h after MCAO-reperfusion compared to that of sham-operated rats. Again, with administration of xanthohumol (0.2 and 0.4 mg/kg), iNOS expression was markedly reduced in MCAO-reperfusion rats (Figure 5B).

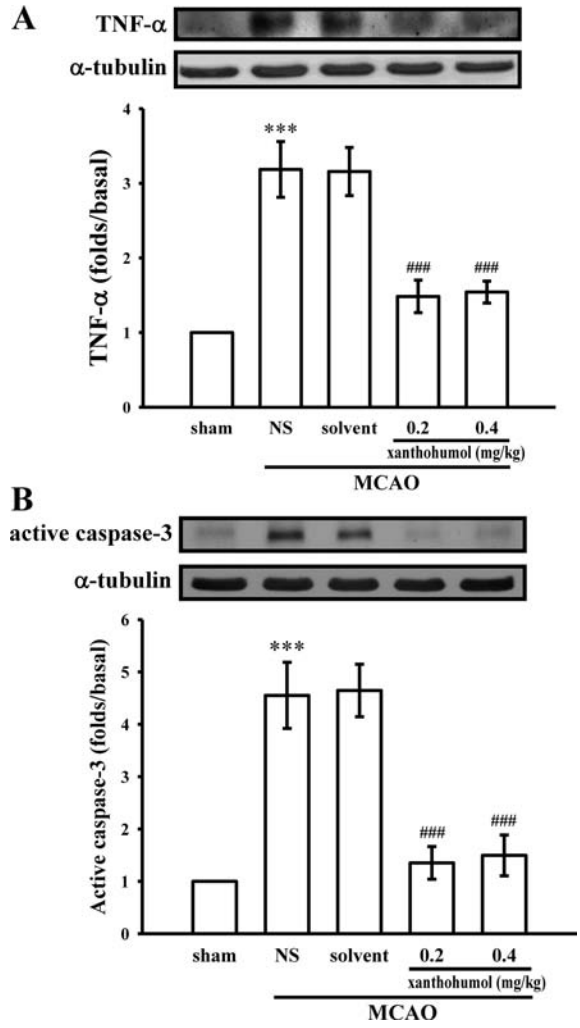

Figure 4. Effects of xanthohumol on the expressions of (A) tumor necrosis factor (TNF)-α and (B) active caspase-3 protein in cerebral homogenates 24 h after middle cerebral artery occlusion (MCAO)-reperfusion injury in rats. Fresh brains from sham-operated, normal saline (NS)-treated, solvent-treated, and xanthohumol (0.2 and 0.4 mg/kg)-treated rats were removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third part of the four sequential parts of the ischemic-injured hemisphere was separately collected, homogenized, and centrifuged. The supernatant (50 μg of protein) was then subjected to SDS–PAGE, and transferred onto membranes for analysis of TNF-α and active caspase-3 expressions. The results are representative examples of five similar experiments. Data are presented as the means ± SEM. $^{***}P < 0.001$ compared to the sham-operated group; $^{\#\#\#}P < 0.001$ compared to the solvent-treated group. Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

Influence of Platelet Aggregation and Hydroxyl Radical (OH•) Formation by Xanthohumol. In Figure 6A, a platelet aggregation curve was induced by collagen in human PRP. Xanthohumol (3, 35, and 70 μM) concentration-dependently exhibited potent activity in inhibiting platelet aggregation stimulated by collagen (1 μg/mL). On the other hand, typical ESR signals of hydroxyl radicals (OH•) were observed in the H_2O_2/NaOH/DMSO system as shown in Figure 6B. Xanthohumol (1.5 and 3 μM) concentration-dependently suppressed hydroxyl radical formation compared to the solvent-treated group. This observation provides in vitro

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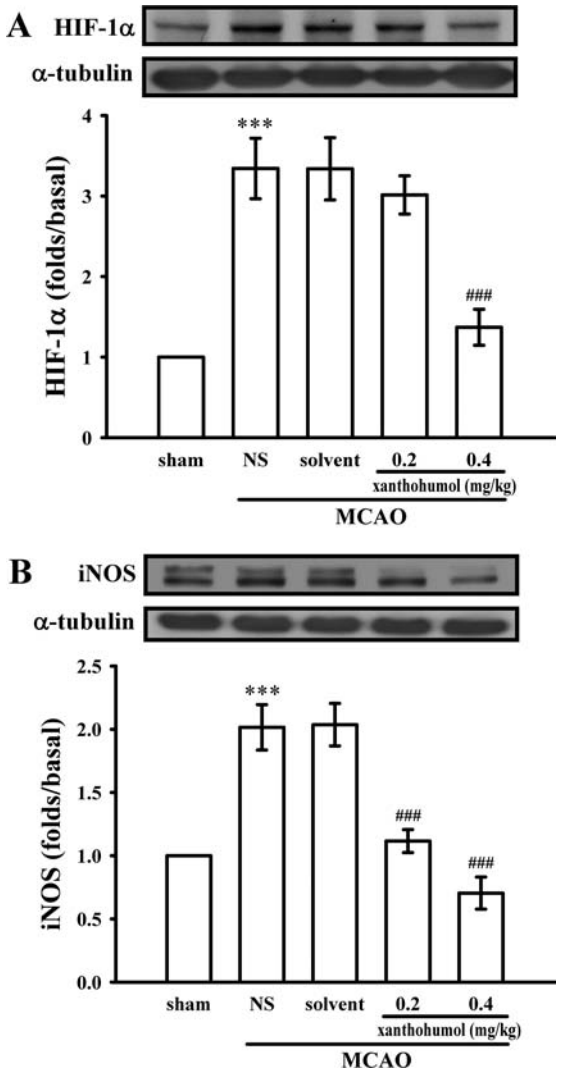

Figure 5. Effects of xanthohumol on the expressions of (A) hypoxia-inducible factor (HIF)-1α and (B) inducible nitric oxide synthase (iNOS) in cerebral homogenates 24 h after middle cerebral artery occlusion (MCAO)-reperfusion injury in rats. Fresh brains from sham-operated, normal saline (NS)-treated, solvent-treated, and xanthohumol (0.2 and 0.4 mg/kg)-treated rats were removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third part of the four sequential parts of the ischemic-injured hemisphere was separately collected, homogenized, and centrifuged. The supernatant (50 μg of protein) was then subjected to SDS–PAGE, and transferred onto membranes for analysis of HIF-1α and iNOS expressions. The results are representative examples of five similar experiments. Data are presented as the means ± SEM. $^{***}P < 0.001$ compared to the sham-operated group; $^{\#\#\#}P < 0.001$ compared to the solvent-treated group. Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

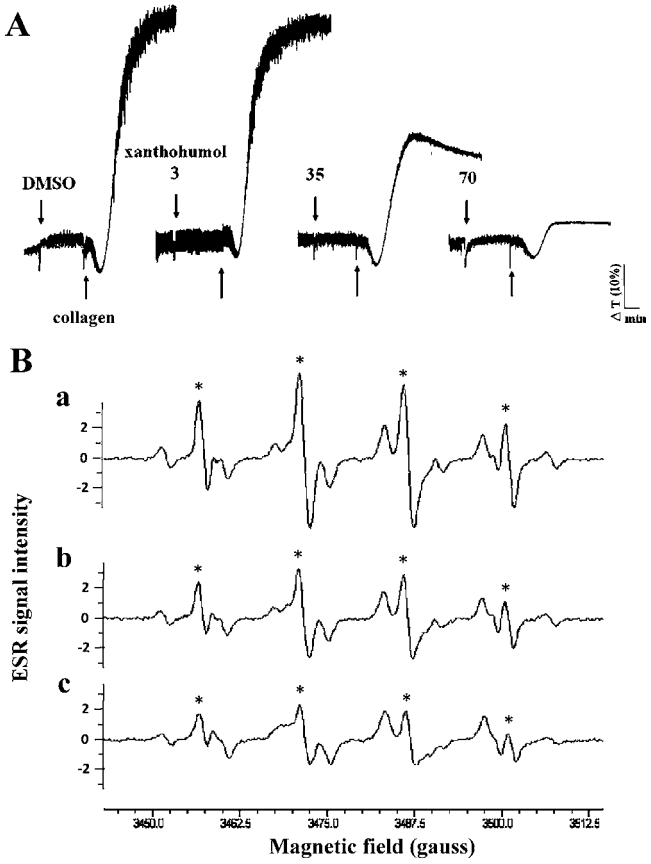

Figure 6. Influence of xanthohumol in platelet aggregation and hydroxyl radical ($OH^{\bullet}$)-scavenging activity. (A) Human platelet-rich plasma was preincubated with various concentrations of xanthohumol (3, 35, and 70 μM) for 3 min, followed by the addition of collagen (1 μg/mL) to trigger platelet aggregation. Profiles are representative examples of four similar experiments. (B) On the other hand, signals of hydroxyl radical peaks were observed in electron spin resonance (ESR) experiments. Typical ESR spectra in the presence of solvent control (a, 0.5% DMSO) or xanthohumol (b, 1.5 μM; c, 3 μM) in the H_2O_2/NaOH/DMSO system as described in Materials and Methods. The spectrum is a representative example of four similar experiments. An asterisk (*) indicates the formation of hydroxyl radicals.

evidence suggesting the neuroprotective effect of xanthohumol may be mediated by free radical-scavenging activity.

DISCUSSION

This study reveals for the first time that, in addition to its well-known anticancer properties, xanthohumol also possesses potent neuroprotective activity. Xanthohumol is the major prenylchalcone of hops (0.1%–1% on a dry weight basis),[23] and beer is known to be a dietary source of xanthohumol and its related prenylflavonoids. The average person in the US consumed 225 mL/day of beer in 2001.[23] Therefore, the daily intake of total prenylflavonoids would be approximately 0.14 mg. Studies on structure–activity relationships (SAR) have shown that xanthohumol and other prenylated chalcones (i.e., 2′,4′,6′,4-tetrahydroxy-3′-geranylchalcone) were participating as the more effective inhibitors of lipid peroxidation in rat liver microsomes than the nonprenylated chalcones (i.e., genistein, quercetin, and chalconaringenin).[24] In generally, the increase of prenyl substituents of chalcones (i.e., 4′-*O*-5′-diprenylxanthohumol) would decrease antioxidant activity in the lipid peroxidation systems, and also cyclization of the prenyl group (i.e., dehydrocycloxanthohumol) resulted in reduction of antioxidant activity. These results demonstrate the importance of prenyl groups in the antioxidant activity of hop chalcones.[24] A study of the pharmacokinetics revealed that the maximal plasma concentration was approximately 180 nM after 4 h of oral administration of 50 mg/kg xanthohumol in rats.[23] Indeed, dietary intake of prenylflavonoids (xanthohumol) through normal beer consumption would not be sufficient to achieve plasma concentrations that could exhibit neuroprotective effectiveness. Ideally, neuroprotection is achieved by long-

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term exposure to nontoxic agents, preferably as part of certain food products or nutritional supplements. Oral treatment with xanthohumol (1000 mg/kg) for 3 weeks in mice showed no signs of toxicity, and a biochemical serum analysis confirmed normal organ function. A recent study has also demonstrated that oral and intravenous injection of xanthohumol at a concentration of 1.86 mg/kg was found safely accumulated in the plasma of rats without causing any toxicity.[25] These data indicate the safe use of xanthohumol as a therapeutic agent or functional nutrient.[26]

Animal models of focal cerebral ischemia, for which MCAO is usually used, reproduce the pattern of ischemic brain damage observed in many human ischemic stroke patients.[27] Cerebral ischemia restricted to the distribution of MCAO gives rise to focal metabolic disturbances that result in infarction, neuronal necrosis, and brain edema.[28] Shiao et al.[29] have reported that some flavonoids isolated from *Flemingia macrophylla* possess potent neuroprotective activity on fibril amyloid β-induced neurotoxicity in neonatal cortical neurons of rats. Our results confirmed that xanthohumol significantly reduced the cerebral infarct volume following focal cerebral ischemia and was effective in improving neurobehavioral deficits.

The present study demonstrates that MCAO-reperfusion injury induces TNF-α, HIF-1α, iNOS, and active caspase-3 protein expressions, which may represent the response of neurons suffering from ischemic insult. TNF-α is one of the key immunomodulatory and proinflammatory cytokines upregulated during brain ischemia.[30] Administration of TNF-α during ischemic brain insult was shown to augment the injury, as evidenced by increased tissue damage and neurological deficits.[30] In addition to inflammation, TNF-α was also shown to be involved in cell apoptosis.[31] On the other hand, the increased HIF-1α protein level observed after MCAO-reperfusion is presumably induced by a loss of the oxygen supply,[32] resulting in a greater extent of binding activity to the iNOS gene and causing a consequent peak in iNOS protein expression. Since the iNOS gene contains the hypoxia-responsive enhancer (HRE) sequence to which HIF-1α binds,[33] results from primary neuronal cultures of cells demonstrated that HIF-1α binds to the iNOS promoter gene under hypoxic conditions. Such binding is associated with an increase in iNOS expression.[34] Furthermore, HIF-1α combined with p53 may promote apoptotic cell death in ischemic areas.[35] In addition, the increased expression of iNOS may also contribute to enhanced neuronal injury, since iNOS knockout mice showed reduced brain damage after ischemia.[36] NO is beneficial as a messenger or modulator, but in conditions such as oxidative stress, it is potentially toxic. Induction of iNOS under inflammatory conditions leads to the production of large amounts of NO for longer periods of time. NO was found to inactivate antioxidative enzymes such as glutathione peroxidase and catalase.[37] There is increasing evidence that NO is involved in mechanisms of cerebral ischemic injury.[38] Altered NO synthesis was implicated in pathophysiological changes of ischemia/reperfusion injury in several key organs. For example, nephrotoxicity and neurotoxicity in animal models of kidney ischemia and brain focal ischemia, respectively, were mediated at least partially by NO, since the toxicity was respectively blocked by an antisense to iNOS and an inhibitor of NOS.[38,39] Microglia play a key role in mediating inflammatory processes in the CNS, which are associated with various neurodegenerative diseases.[28] Lee et al.[17] recently reported that xanthohumol inhibited inflammatory mediators such as TNF-α and NO formation in LPS-stimulated microglia.

Several apoptosis-related proteins, including caspases-9 and -3, are strongly expressed after ischemic injury. In addition, hypoxia may cause HIF-1α to bind to p53 in order to stabilize it, and also activates the expression of various genes including Bax (a proapoptotic member of the Bcl-2 family).[40] Bax is translocated to mitochondria where it releases cytochrome *c* into the cytosol to interact with apoptotic protease activating factor (Apaf)-1 to activate caspase-9, which in turn activates downstream caspases, such as caspase-3.[41] In the present study, we showed that elevation of active caspase-3 and iNOS expressions occurred in the same time frame as TNF-α and HIF-1α expressions after ischemic injury, and these expressions were significantly suppressed by xanthohumol.

Platelet aggregation plays a pathophysiological role in cerebrovascular disorders. Inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. We previously demonstrated that endothelial cell injury induces platelet aggregation and adhesion to vessel walls.[42] In the present study, xanthohumol markedly inhibited platelet aggregation in human platelets, indicating that this effect may be involved in xanthohumol-mediated neuroprotection against ischemic brain injury.

The ROS-scavenging activity of xanthohumol was reported by Gerhäuser et al.;[11] they found that xanthohumol was about 9-fold more potent than Trolox at a concentration of 1 μM at scavenging hydroxyl and peroxyl radicals as analyzed by an indirect method of oxygen radical antioxidant capacity (ORAC) assay. In the present study, we also examined whether xanthohumol has direct free radical-scavenging activity in a cell-free (H_2O_2/NaOH/DMSO) system, and it was assumed that superoxide anions and hydroxyl radicals were generated from the degradation of hydrogen peroxide.[22] The superoxide anion changes into a hydroxyl radical by the catalytic action of contaminating trace iron, so that the amount of hydroxyl radicals is consequently relatively larger than that of superoxide anions. Therefore, the hydroxyl radical-scavenging activity of xanthohumol could be directly evaluated by the ESR experiment. Those results suggested that neuroprotection by xanthohumol may be involved, at least partly, in the inhibition of free radical formation.

In conclusion, the most important findings of this study suggest that the neuroprotective effect of xanthohumol on cerebral ischemic damage in MCAO-reperfusion rats is probably mediated by inhibition of inflammatory responses (i.e., HIF-1α, iNOS expression, and free radical formation), apoptosis (i.e., TNF-α, active caspase-3), and platelet activation, resulting in a reduction of the infarct volume and improvement of neurobehavior in cerebral ischemic rats. The rationale for the use of xanthohumol is based on the fact that multiple deleterious processes in different cell types of organelles are initiated during ischemia-reperfusion injury which ultimately synergistically moves toward irreversible injury. Therefore, treatment using xanthohumol is not limited to one factor but involves many mechanisms, most of which may be interrelated. For example, xanthohumol-induced neuroprotection is related to inflammation, NO, and apoptosis, and many of those factors (such as iNOS, active caspase-3, etc.) are related to HIF-1α. We speculate that correlating these molecules and morphological changes may lead to neurobehavioral improvement in patients; thus, the novel role of xanthohumol in neuroprotection may represent high therapeutic potential for treating or preventing

such diseases, in addition to it originally being considered as a chemopreventive agent.

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Notes

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ABBREVIATIONS USED

BSA, bovine serum albumin; ESR, electron spin resonance; $OH^{\bullet}$, hydroxyl radical; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; PRP, platelet-rich plasma; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide anions; TPA, tissue plasminogen activator; TNF-α, tumor necrosis factor-α

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