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# Methyleugenol reduces cerebral ischemic injury by suppression of oxidative injury and inflammation

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**Abstract**

The present study tested the cytoprotective effect of methyleugenol in an *in vivo* ischemia model (i.e. middle cerebral artery occlusion (MCAO) for 1.5 h and subsequent reperfusion for 24 h) and further investigated its mechanism of action in *in vitro* cerebral ischemic models. When applied shortly after reperfusion, methyleugenol largely reduced cerebral ischemic injury. Methyleugenol decreased the caspase-3 activation and death of cultured cerebral cortical neurons caused by oxygen-glucose deprivation (OGD) for 1 h and subsequent re-oxygenation for 24 h. Methyleugenol markedly reduced superoxide generation in the ischemic brain and decreased the intracellular oxidative stress caused by OGD/re-oxygenation. It was found that methyleugenol elevated the activities of superoxide dismutase and catalase. Further, methyleugenol inhibited the production of nitric oxide and decreased the protein expression of inducible nitric oxide synthase. Methyleugenol down-regulated the production of pro-inflammatory cytokines in the ischemic brain as well as in immunostimulated mixed glial cells. The results indicate that methyleugenol could be useful for the treatment of ischemia/inflammation-related diseases.

**Keywords:** Methyleugenol, ischemic injury, OGD, ROS, NO





## Introduction

Cerebral hypoxia/ischemia is one of the most important life-threatening causes of brain injury. The ischemic injury is evoked by many inter-related causes such as excessive stimulation of excitatory amino acid receptors, intracellular calcium accumulation, energy failure, apoptotic cell death, lipid peroxidation, and free radical generation [1].

The level of reactive oxygen species (ROS) in normal cells is tightly regulated by biological antioxidants and antioxidant enzymes [2]. In pathological conditions, the generation of oxidants exceeds the intracellular antioxidant capacity, resulting in oxidative damages to proteins, lipids and DNA. Thus, ROS has been closely recognized as a key mediator associated with neurodegeneration induced by a variety of insults including excitotoxicity, amyloid beta, ischemia and nerve growth factor withdrawal [3].

Methyleugenol (4-allyl-1,2-dimethoxybenzene), an alkenylbenzene compound, is present as a component of leaf and floral essential oils from many plant species [4]. A structurally similar compound eugenol is known to act as an anti-oxidant [5], anti-lipid peroxidant [6], anti-allergic [7], anti-inflammatory [8], anesthetic [9] and anti-fungal agent [10]. Eugenol also protects neurons from excitotoxic and oxidative injury via modulation of both NMDA receptor and superoxide radicals [11,12]. Other similar compounds (demethyldiisoeugenol and isoeugenol) can also scavenge superoxide anion generated by xanthine/xanthine oxidase and peroxyl radical [13,14].

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Methyleugenol has many biological properties including anti-allergic [7], anti-anaphylaxis [15] and anti-nociceptive [16] effects. However, little study has been done of the anti-ischemic and anti-inflammatory activities of methyleugenol. Thus, in the present study we investigated those activities of methyleugenol in *in vivo* as well as *in vitro* experimental models.

## Materials and methods

### *Animals*

Male Sprague-Dawley rats weighing between 260–270 g were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12-h light/dark cycle with *ad libitum* access to food and water. Rats were acclimated to their environment for 5 days before use for experiments. All experimental procedures using animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Korea University College of Medicine.

### *Focal cerebral ischemia model*

Rats were initially anaesthetized with 3.0% isoflurane in a 70% $N_2O$ and 30% $O_2$ (v/v) mixture via a facemask. Anaesthesia was maintained with 2% isoflurane. A rectal temperature probe was introduced and a heating pad maintained the body temperature at 37°C throughout the surgery period. Focal cerebral ischemia was achieved by right-sided endovascular middle cerebral artery occlusion (MCAO) [17]. Briefly, the right carotid arteries were exposed through a midline cervical incision. The right external carotid artery (ECA) was dissected free and isolated distally by coagulating its branches and placing a distal ligation before transection. A piece of 3-0 monofilament nylon suture (Ethicon, Johnson-Johnson, Brussels, Belgium), with its tip rounded by gentle heating and coated by 0.1% (w/v) poly-L-lysine (Sigma-Aldrich, St. Louis, MO), was inserted into the lumen of the right ECA stump and gently advanced 17.5 mm into the internal carotid artery (ICA) from the bifurcation to occlude the ostium of MCAO. After 1.5 h of ischemia, the suture was pulled back and the animal was allowed to recover. Sham-operated controls were subjected to the same surgical procedures except that common carotid arteries were not occluded. Body temperature was monitored and maintained at 37°C ± 0.3°C during surgery and during the immediate post-operative period until the animals recovered fully from anaesthesia. Methyleugenol (100 mg/kg) was intraperitoneally injected 2 h after starting MCAO (i.e. 30 min after starting reperfusion).

### *Measurement of infarct volume*

Rats were anaesthetized with chloral hydrate and decapitated at 3 h and 1 day after MCAO. Rat brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA). The brain slices were then incubated in 2% triphenyltetrazolium chloride (TTC; Sigma-Aldrich) at 37°C for 30 min to reveal the ischemic infarction. After TTC reaction, the brain slices were fixed with 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer (PB) for 1 day and subsequently cryoprotected in PB containing 30% sucrose at 4°C for 2 days. The cross-sectional area of infarction between the bregma levels of +4 mm (anterior) and −6 mm (posterior) was determined with a computer-assisted image analysis program (OPTIMAS 5.1, BioScan). On each slice, brain infarct size was measured manually by outlining the margins of infarct areas and the infarct volume was calculated according to the slice thickness of 2 mm per section. Each side of the brain slices was measured separately and the mean values were calculated. The total volume of infarction was determined by integrating six chosen sections and expressed as a percentage of the total brain volume. Because post-ischemic brain oedema will increase brain volume in the infarct area, the corrected infarct volumes were calculated to compensate for brain oedema, as previously described by Yang et al. [18]. Thereafter the tissues were frozen and cut into 10 or 30 μm coronal sections on a cryostat (Leica 3050, Leica, Germany) and stored at −20°C.

### *Detection of oxidative cellular injury after reperfusion*

To confirm the occurrence of oxidative stress, *in situ* detection of oxidized hydroethidine (HEt) was performed 24 h after reperfusion by modifying a previously described method [19]. Because HEt is oxidized to ethidium mainly by the superoxide anion radical [20], detection of oxidized HEt has been used to show oxidative stress level. HEt (200 μg in 1% dimethysulphoxide-containing PB; Molecular Probes, Eugene, OR) was administrated intravenously 5 min before the induction of ischemia. The animals were sacrificed 24 h after reperfusion by transcardial perfusion with 200 ml of 0.9% saline and 200 ml of 4% paraformaldehyde in 0.1 M PBS. After post-fixation overnight, the brains were sectioned with a cryostat (Leica 1850, Leica, Germany) into a thickness of 8 μm at the level of the mid striatum. The sections were then counterstained with Hoechst 33258 (Molecular Probes) for 20 min. These sections were observed with a microscope under fluorescent light (Olympus BX 51, Olympus co, Japan). Intensity and expression patterns of the oxidized HEt were analysed after taking photographs with a digital camera (spot basic 4.6, USA) by double exposure to oxidized HEt and Hoechst 33258.

*Immunohistochemistry*

Immunohistochemical reaction was performed using ABC methods. The sections were treated with 0.3% hydroperoxide in PBS (pH 7.3) for 30 min and then incubated in 10% normal horse serum-supplemented PBS for 30 min. The sections were then incubated at room temperature with primary antibodies in PBS containing 0.3% triton X-100 and 1% normal horse serum overnight. Primary antibodies were used at the following dilutions: rabbit anti-IL-1β (1:200, Abbiotec, San Diego, CA); rabbit anti-TNF-α (1:200, Abbiotec). After washing three times for 10 min each with PBS, sections were incubated sequentially with biotinylated anti-mouse IgG, anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and then with peroxidase-conjugated streptavidin (Vector Laboratories), diluted 1:200 in the same solution as the primary antiserum. To establish the specificity of each primary antibody, control sections were similarly treated in the absence of primary antibodies. The antigens were visualized by 5 min of incubation in 0.05 M Tris-HCl buffer containing 0.02% 3,3-diaminobenzidine and 0.0045% hydrogen peroxide at 37°C. Finally, all sections were mounted on slides coated with gelatin solution, dehydrated, coverslipped, and then analysed under a bright-field microscope (Olympus).

*Cell culture*

Primary cortical neuronal cultures were prepared from embryonic 17~18 days old foetal Sprague-Dawley rats. In brief, cerebral cortices were dissected from the brain and then freed of meninges and blood vessels. Cerebral cortices were chopped in ice-cold Hanks' Balanced Salt Solution (HBSS; WelGENE Inc., Seoul, Korea) and dissociated by trituration through a Pasteur pipette. Cerebral cortices were further dissociated by repeated pipetting and filtered through a nylon sieve. Cell suspension (cell number: $5 \times 10^5$ cells/ml) was added onto the culture plates pre-coated with poly-D-lysine (100 μg/ml; Sigma-Aldrich, St. Louis, MO) and laminin (4 μg/ml; Millipore, Bedford, MA). Cells were maintained in 10% foetal bovine serum (FBS; Hyclon, Logan, UT)-supplemented Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Seoul, Korea) in humidified 95% air/5% $CO_2$ at 37°C. Three days later, cytosine arabinoside (5 μM; Sigma-Aldrich) was added for 1 day to block non-neuronal cell division. Subsequent media replacement was carried out twice a week. Experiments were performed on cultures 14–16 days after initial plating. In general, neurons retained in culture for 14–16 days are considered as a model of 'mature neurons' [21].

Mixed glial cultures were prepared from the prefrontal cortices of 1-day-old Sprague-Dawley rat pups. Tissue free of meninges was collected by low speed centrifugation and then dissociated by trituration through a Pasteur pipette. Cells were then plated onto poly-D-lysine (1 μg/ml; Sigma-Aldrich, St. Louis, MO)-coated 75-$cm^2$ culture bottles and kept for 1 week in Minimum Essential Medium (MEM; WelGENE Inc., Seoul, Korea) supplemented with 10% FBS. Mixed glial cells were then trypsinized, washed and replated (cell number: $1 \times 10^4$ cells/ml) in the growth medium onto poly-D-lysine (10 μg/ml)-coated 24-well plates. Cells were used for the experiments 7–9 days later.

*Oxygen-glucose deprivation followed by re-oxygenation*

For *in vitro* hypoxic/ischemic insult, cells were placed in an anaerobic chamber (partial pressure of oxygen $< 2$ mmHg), and the culture medium was replaced with a glucose-free DMEM bubbled with an anaerobic gas mix (95% $N_2$, 5% $CO_2$) for 30 min to remove residual oxygen. Cells were left in an anaerobic chamber at 37°C for a 60 min period to produce oxygen deprivation. Control cells, not exposed to OGD, were maintained in glucose (20 mM)-containing DMEM aerated with an aerobic gas mix (95% air, 5% $CO_2$). Oxygen-glucose deprivation was stopped by replacing the exposure solution with oxygenated DMEM supplemented with 20 mM glucose and returning the cells to the incubator under normoxic conditions. Cells were treated with methyleugenol at 30 min before/during OGD/re-oxygenation.

*Assessment of cell injury or death*

Cell injury or death was quantified by measuring the amount of lactate dehydrogenase (LDH) released into the bathing medium [22]. Activity of LDH was measured using a diagnostic kit (Sigma-Aldrich, St. Louis, MO). Cell viability was expressed as a percentage of total LDH, which was measured in sister cultures frozen and thawed after the experiments.

*Measurement of caspase-3 protease activity*

Cortical neurons were exposed to OGD for 3 h and caspase-3-like activity was measured in lysates of cortical neurons using the caspase-3 fluorogenic peptide substrates (Calbiochem, Darmstadt, Germany) following the instructions of the manufacturer. Cells were harvested and lysed in a lysis buffer containing 10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 2 mM EGTA, 2 mM EDTA, 1 mM PMSF and protease inhibitor cocktail. Lysates were centrifuged at 12 000 *g* for 10 min at 4°C and the supernatants were collected. The supernatants (10 μl) were incubated in a 96-well plate with 90 μl of assay buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10% sucrose) containing

Table 1. Conditions for PCR of target genes.

| Gene | Primer sequence | No. cycles | Product size | Accession no. |
|---|---|---|---|---|
| **iNOS** | For 5′-ACA CAG CCT CAG AGT CCT TC-3′<br>Rev 5′-GAA CTG AGG GTA CAT GCT GG-3′ | **28** | **593** | **NM_012611** |
| **IL-10** | For 5′-AGT GGA GCA GGT GAA GAA TG-3′<br>Rev 5′-CTG GTT TCT CTT CCC AAG AC-3′ | **32** | **599** | **NM_012854** |
| **TGF-β1** | For 5′-CTG AGT GGC TGT CTT TTG AC-3′<br>Rev 5′-TTG CGA CCC ACG TAG TAG AC-3′ | **26** | **540** | **NM_021578** |
| **IL-1**β | For 5′-GTG TGG ATC CCA AAC AAT AC-3′<br>Rev 5′-CCA TAC ACA CGG ACA ACT AG-3′ | **26** | **555** | **NM_031512** |
| **IL-6** | For 5′-TGT TCT CAG GGA GAT CTT GG-3′<br>Rev 5′-TCT GAC CAC AGT GAG GAA TG-3′ | **28** | **517** | **NM_012589** |
| **TNF-α** | For 5′-CAC GCT CTT CTG TCT ACT GA-3′<br>Rev 5′-GGA CTC CGT GAT GTC TAA GT-3′ | **27** | **541** | **AJ002278** |
| **GAPDH** | For 5′-ACT CCC TCA AGA TTG TCA GC-3′<br>Rev 5′-CAT ACT TGG CAG GTT TCT CC-3′ | **25** | **342** | **NM_017008** |

iNOS, inducible nitric oxide synthase; IL-1β, Interlukin 1 beta; IL-6, Interlukin 6; IL-10, Interlukin 10; TGF-β1, Transforming growth factor-beta1; TNF-α, Tumor necrosis factor-alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase For, forward; Rev, reverse.



30 μM caspase substrate (caspase-3 substrate Ac-DEVD-AMC; Calbiochem, Darmstadt, Germany). AMC from Ac-DEVD-AMC was measured on a microplate fluorescence reader (SpectraMax GeminiEM; Molecular Devices) with an excitation wavelength of 360 nm and an emission wavelength of 480 nm.

### *Measurement of free radicals*

Cells were exposed to OGD/re-oxygenation and immunostimulation in the absence or presence of methyleugenol. Cells were loaded with 2, 7-dihydrodichlorofluorescein diacetate (DCF-DA, 30 μM; Calbiochem, Darmstadt, Germany) in PBS for 20 min and then rinsed with the same solution. After a 20-min incubation at room temperature, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm (slit widths 20 and 25 nm, respectively) using a fluorescence microplate reader (FL600, Bio-tek Instruments, Inc., Winooski, VT). DCF-DA diffuses through cell membranes and is subsequently enzymatically deacetylated by intracellular esterases to the non-fluorescent DCF-H. Free radicals such as Peroxynitrite and $H_2O_2$ effectively convert DCF-H to the highly fluorescent DCF [23]. Fluorescence intensities were corrected for autofluorescence (i.e. fluorescence of cells not loaded with DCF-DA).

### *1,1-diphenyl-2- picrylhydrazyl (DPPH) assay*

The free radical scavenging capacity of methyleugenol was analysed by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich) assay. An aliquot of each compound tested at various concentrations (100, 50, 25, 10, 1 and 0.1 μg/ml in ethanol) was mixed with 23.6 μg/ml of DPPH solution in ethanol. After incubation of the mixture for 30 min, the absorbance of the remaining DPPH was determined colourimetrically at 517 nm. The scavenging activities were

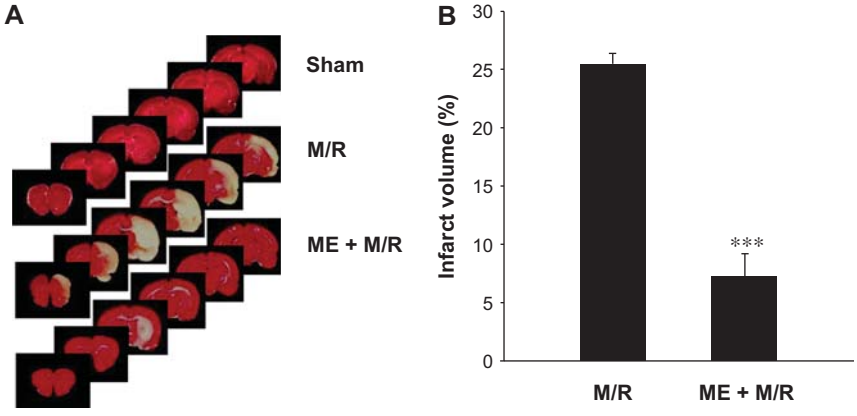


Figure 1. Methyleugenol reduces cerebral infarction. (A) Representative TTC-stained coronal brain sections with six slices (2 mm-thick) each between 4–16 mm from the frontal pole. Rats were intraperitoneally administered with methyleugenol (ME), as described in the Materials and methods. Rats were exposed to MCAO (M) for 1.5 h and reperfusion (R) for 24 h. (B) The graph shows percentage changes of infarct volume. Data are presented as mean ± SD from 12 rats. $^{***}p < 0.001$, compared with the infarct volume obtained in saline M/R group.

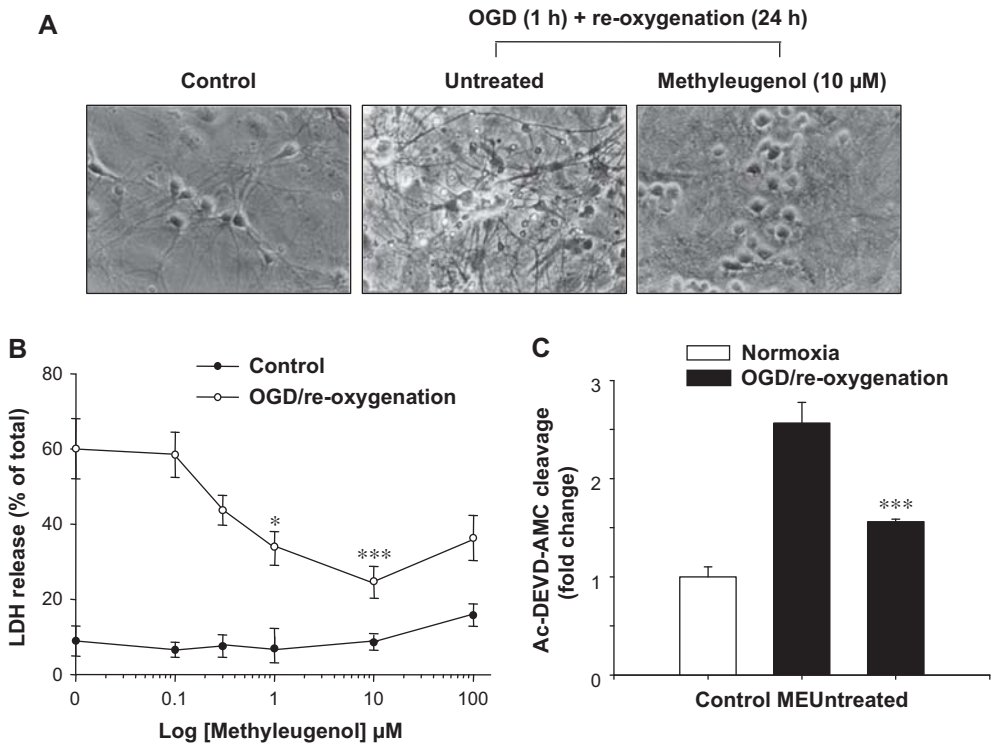


Figure 2. Methyleugenol protects cortical neurons from OGD/re-oxygenation toxicity. Neuronal cells were exposed to 1 h OGD and 24 h re-oxygenation in the absence or presence of methyleugenol (ME). (A, B) Cell injury or death was assessed by morphological observations (A) or by measuring the LDH release into the bathing medium (B). (C) Cells were harvested, lysed and then the activity of caspase 3 was determined by DEVD-AMC cleavage assay. Data are expressed as mean ± SEM from five separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with OGD group.

expressed as a percentage of the absorbance of the control DPPH solution. Ascorbic acid (Vit C; Sigma-Aldrich) was used as standard. The results are expressed as mean of at least three independent experiments. Results were expressed as percentage activity. The $IC_{50}$ values were calculated by use of the Litchifield and Wilcoxon [24] test.

### *Antioxidant enzyme activity assay*

For SOD activity assay, cells were washed with cold PBS and collected by centrifugation at 3000 rpm for 10 min. Cell pellets were lysed in cold 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, 70 mM sucrose and 0.3% Triton X-100. The lysate was recentrifuged at 12 000 rpm for 5 min. Total SOD and Mn-SOD activities in the supernatant were determined using a superoxide dismutase assay kit (Cayman Chemical, CA). For Mn-SOD activity assay, 2 mM potassium cyanide was added to the lysate to inhibit Cu/Zn-SOD. For catalase activity assay, cells were washed with cold PBS and then lysed in PBS containing 1% Triton X-100. The lysate was centrifuged at 12 000 rpm for 15 min. Catalase activity in the supernatant was determined using a catalase assay kit (Cayman Chemical, CA). The results obtained from the antioxidant enzyme activity assay were expressed as the percentage of control.

### *Immunostimulation and NO production*

Primary rat mixed glial cells were treated with IFN-γ (100 U/ml; Calbiochem, Darmstadt, Germany) and lipopolysaccharide (LPS, 1 μg/ml; Sigma-Aldrich) for 48 h. NO production from immunostimulated mixed glial cells was determined by measuring nitrite, a stable oxidation product of NO, as described previously [25]. In brief, nitrite levels were determined by adding the Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) to the medium. After 10 min, the absorbance at 550 nm was determined using a UV spectrophotometer (Beckman DU-650, Fullerton, CA).

### *Immunocytochemistry*

Cells were fixed with the fixation solution (4% paraformaldehyde, 4% sucrose, 0.01 M PBS and 50 mM HEPES, pH 7.5) at 25°C for 15 min. Cells were then washed three times for 5 min in PBS and permeabilized with a solution containing 3% normal horse serum and 0.3% Triton X-100 in PBS for 30 min. Cells were washed twice in PBS and incubated with a rabbit polyclonal anti-iNOS antibody (diluted 1:500; Santa Cruz, CA) in PBS containing 0.3% triton X-100 and 3% normal horse serum overnight at

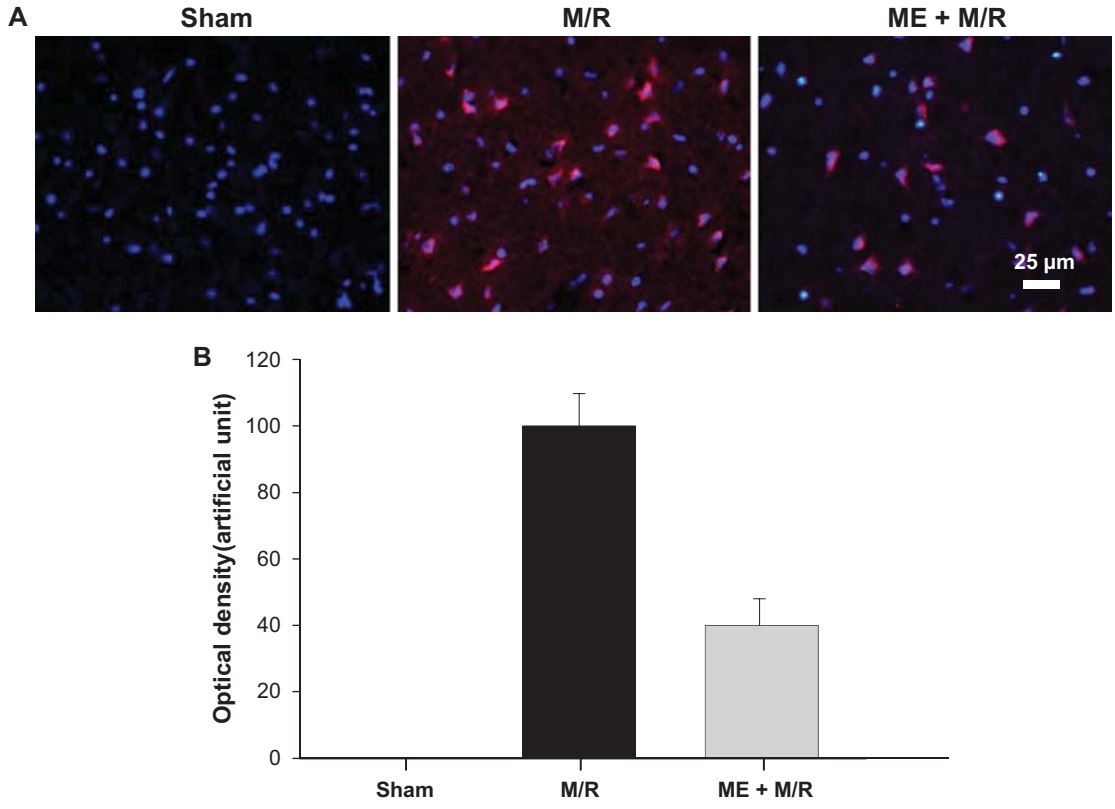


Figure 3. Methyleugenol prevents superoxide generation in ischemic brain lesion. (A) As described in the Materials and methods, HEt was administrated intravenously 5 min before the induction of ischemia. Brain slices were prepared 24 h after reperfusion and then counterstained with Hoechst 33258 (blue). Intensity and expression patterns of the oxidized HEt (red) were analysed after taking photographs with a digital camera (spot basic 4.6, USA). (B) Histogram reveals a quantitative analysis of mean signal intensity for hydroethidine stained lesion.



4°C. After washing twice for 10 min with PBS, cells were incubated sequentially, in biotinylated goat anti-rabbit IgG (Vector) and peroxidase-conjugated streptavidin (Vector), diluted 1:200 in the same solution as the primary antiserum. Between the incubations, cells were washed with PBS twice for 10 min. Cells were visualized with 3,3-diaminobenzidine (0.5 mg/ml, with or without 0.2 mg/ml nickel chloride) in 0.1 M Tris buffer.

### *Reverse transcription polymerase chain reaction*

Total RNA was isolated from cultured primary cells with RNeasy mini kit from Qiagen (Hilden, Ger-

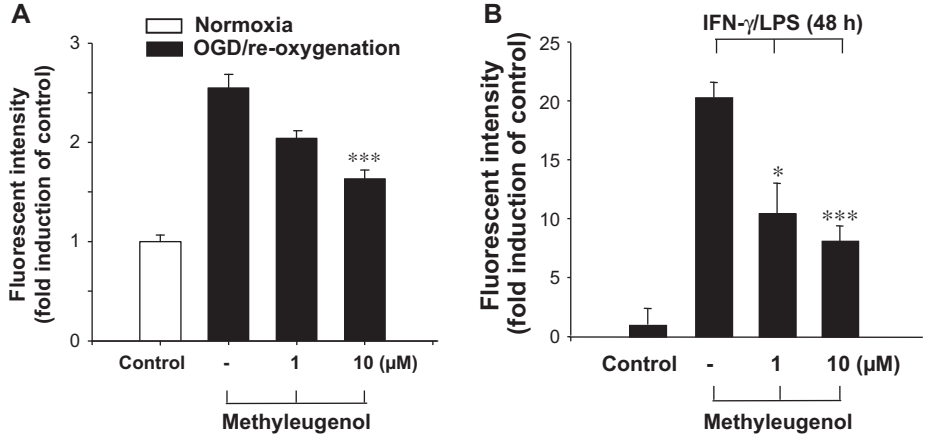


Figure 4. Methyleugenol suppresses ROS production in mixed glial cells exposed to OGD/re-oxygenation or immunostimulation. Cells were exposed to OGD/re-oxygenation (A) or immunostimulation (B) in the absence or presence of methyleugenol. Intracellular ROS levels were measured by using DCF-DA. The graphs show fluorescent intensities in each experimental condition. Data are presented as mean ± SEM from three separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with untreated group.

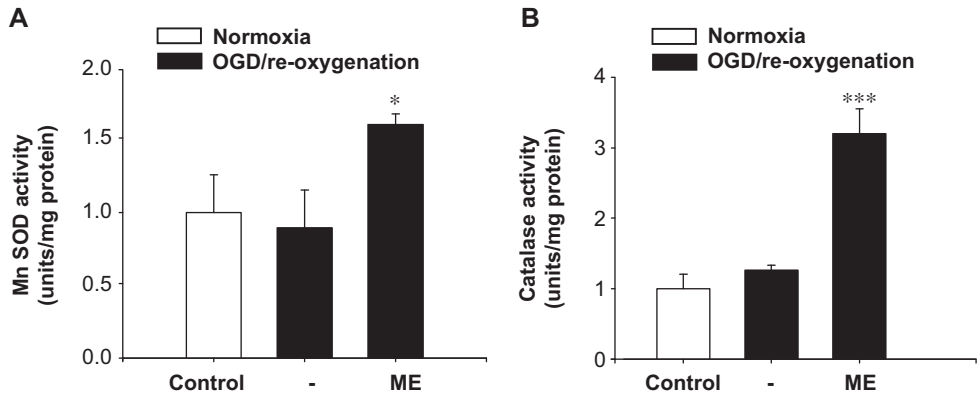


Figure 5. Methyleugenol up-regulates antioxidant enzymes. Neuronal cells were exposed to OGD and re-oxygenation in the absence or presence of methyleugenol. Cells were harvested and lysed for Mn-SOD (A) and catalase (B) assay. The activities of Mn-SOD and catalase were measured with a Cayman assay kit. Data are presented as mean ± SEM from three separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with OGD group.



many). One microgram of RNA was reverse-transcribed using an Omniscript RT kit (Qiagen, Hilden, Germany) and the resulting cDNA was amplified by PCR using a HotStarTaq Master Mix kit (Qiagen, Hilden, Germany). PCR cycles were performed as follows: denaturation at 94°C for 50 s, annealing at 57°C for 50 s and extension at 72°C for 60 s for each cycle of target genes; finally, additional extension at

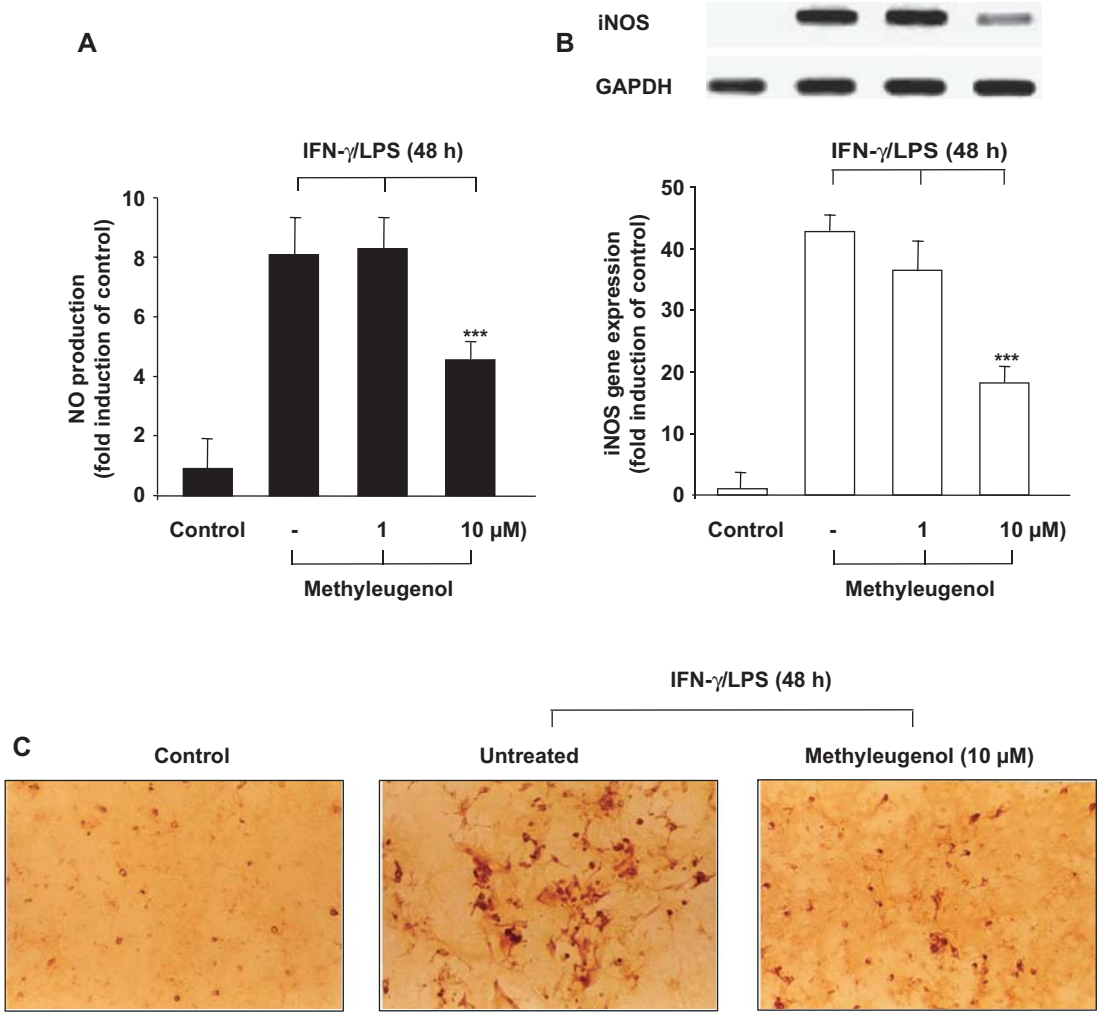


Figure 6. Methyleugenol attenuates iNOS expression and NO production. (A) Mixed glial cells were stimulated with IFN-γ/LPS for 48 h. Methyleugenol suppressed the NO production in IFN-γ/LPS-treated glial cells. (B and C) RT-PCR and immunocytochemistry. Data are presented as mean ± SEM from three separate experiments. $^{***}p < 0.001$, significantly different from the cells treated with IFN-γ/LPS in the absence of methyleugenol.

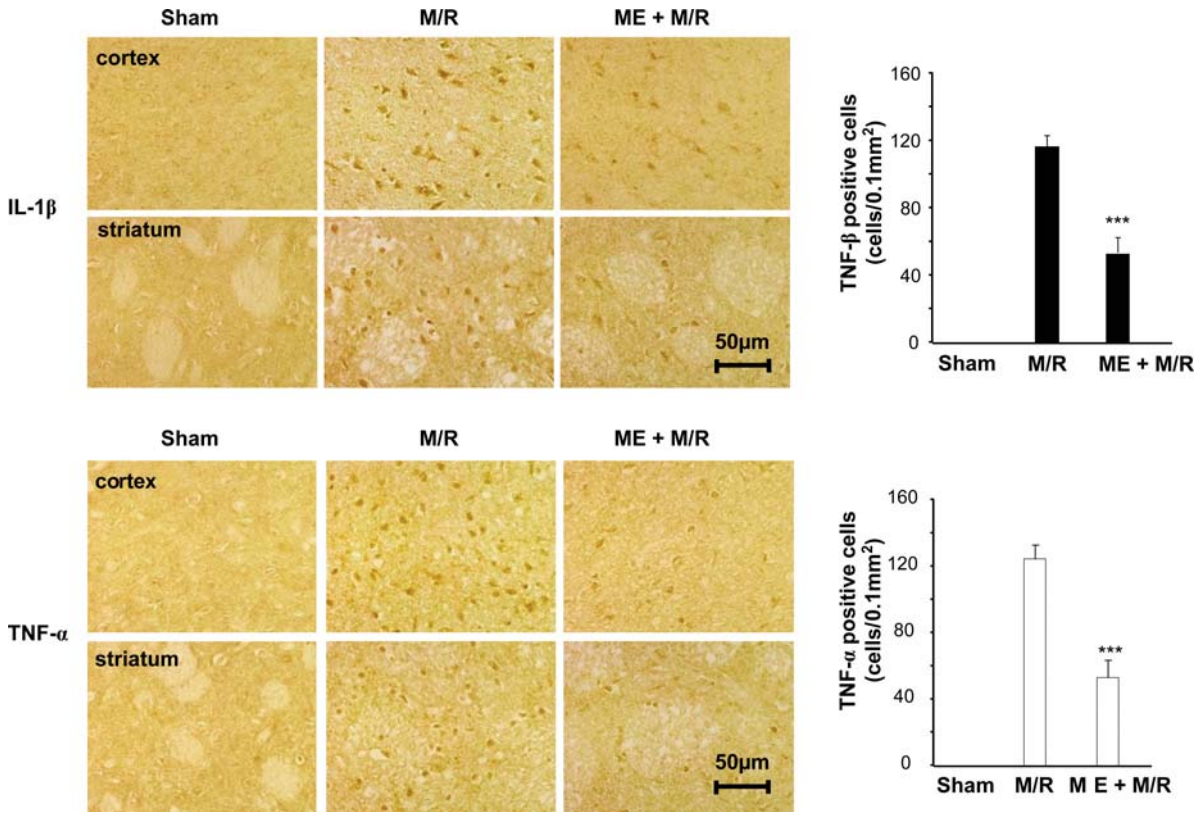


Figure 7. Methyleugenol decreases expressions of IL-1β and TNF-α in ischemic brain lesion. Immunohistochemical studies were done with anti-IL-1β- or TNF-α antibodies, as described in the Materials and methods. For quantitative analysis, IL-1β- or TNF-α-positive cells were counted at three random selected regions per independent experiment. $n = 3$. $^{***}p < 0.001$, compared with MCAO/reperfusion group.

72°C for 10 min. Target genes primers, each cycle of target genes, product size and accession number are shown in Table I.

### *Statistical analysis*

Data are expressed as the means ± SD or SEM and analysed for statistical significance using repeated measures ANOVA by running the SAS Window v.9.1. program. For multiple comparisons, post-hoc Scheffe's test was performed. A $p$-value $< 0.05$ was considered significant.

## Results

First, we determined physiological parameters, as we described before [26]. Intraperitoneal treatment of methyleugenol did not change physiological parameters such as mean arterial pressure, pH, arterial partial $CO_2$ and $O_2$ pressures and blood glucose concentration (data not shown). In rat brain treated with 1.5-h MCAO/24-h reperfusion, methyleugenol largely reduced the infarct (Figure 1) and cerebral oedema (data not shown).

We next tested whether methyleugenol protected cells from OGD/re-oxygenation-evoked death of cortical neurons. OGD/re-oxygenation caused pathological alterations in cell morphology (Figure 2A) and increased the release of LDH in cultured cortical neurons (Figure 2B). Methyleugenol markedly decreased the OGD/re-oxygenation-evoked LDH release (Figure 2B). In general, apoptosis is not simply identified by morphological changes. Caspases are key mediators of cell death and caspase-3 is an executioner for the death programme in cortical neurons in response to various noxious stimuli [27]. Thus, we checked the activation of caspase-3 after OGD/re-oxygenation by using a fluorogenic peptide substrate (Ac-DEVD-AMC) for caspase-3. In the present study, methyleugenol significantly reduced the caspase-3 activity, which was enhanced by OGD/re-oxygenation (Figure 2C).

We hypothesized that methyleugenol treatment could protect rat brain cells from oxidative injuries due to its anti-oxidant activity. Thus, we examined the free radical scavenging activity of methyleugenol by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical donor. We found that methyleugenol has a free radical scavenging activity: the $IC_{50}$ value of methyleugenol and vitamin C were 13.7 and 3.6 μM, respectively (data not shown). Next, we measured the level of superoxide anion generation by *in situ* detection of oxidized HEt. MCAO (1.5 h)/reperfusion (24 h) highly increased the level of oxidized HEt in the

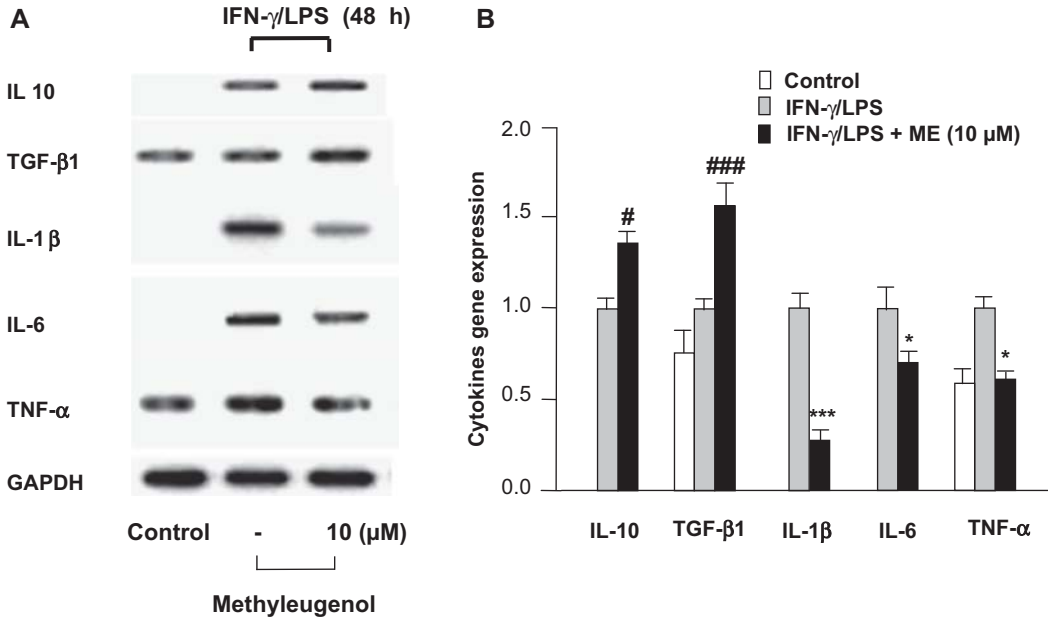


Figure 8. Methyleugenol alters the mRNA expression levels of pro- and anti-inflammatory cytokines. (A) Mixed glial cells were stimulated with IFN-γ/LPS for 48 h. The levels of mRNA expression were measured by RT-PCR. (B) The quantification of mRNA expression levels. For quantification, the mRNA expression level was normalized with respect to GAPDH and then expressed as relative fold changes in comparison to appropriate LPS/IFN-γ-treated groups, which were assigned to 1.0. Data are presented as mean ± SEM from five separate experiments. $^{\#,*}p < 0.05$, $^{\#\#\#,\ ***}p < 0.001$; significantly different from the appropriate groups treated with IFN-γ/LPS in the absence of methyleugenol.

ischemic lesion. However, post-ischemic treatment of methyleugenol largely reduced the level of superoxide anion (Figure 3). Our further studies showed that methyleugenol significantly reduced the ROS levels in cells exposed to the OGD/re-oxygenation (Figure 4A) or activated with LPS/IFN-γ (Figure 4B). The reduction of ROS level by methyleugenol could be due to direct scavenging of radicals by methyleugenol. However, it is also possible that methyeugenol removes radicals indirectly by up-regulating the activities of antioxidant enzymes. Thus, we further tested this possibility. As shown in Figure 5, treatment with methyleugenol largely increased Mn-SOD and catalase activities.

The up-regulated expression of iNOS has been associated with brain ischemic injury [28]. Methyleugenol suppressed the NO production and iNOS expression in immunostimulated glial cells (Figure 6). In the delayed stage of ischemic stroke, production of inflammatory cytokines are also crucial for brain tissue damage [29]. Thus, we further examined whether methyleugenol could modulate the production of inflammatory cytokines. Immunohistochemical studies showed that MCAO/reperfusion highly increased the protein expression levels of IL-1β and TNF-α. However, methyleugenol significantly reduced their expressions in ischemic brain lesion (Figure 7). Our further studies showed that methyleugenol decreased the mRNA expressions of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in immunostimulated glial cells. Reversely, however, methyleugenol increased the mRNA expressions of anti-inflammatory cytokines such as IL-10 and TGF-β (Figure 8).

## Discussion

Ischemia/hypoxia-evoked neuronal cell death is caused by many different but inter-related mechanisms such as excessive stimulation of excitatory amino acid receptors, intracellular calcium accumulation, energy failure, caspase-dependant cell death, and oxidative stress. Also, inflammation is the other devastating continuous response in brain tissue damage. In the present study, we show clear evidence that methyleugenol has multi-functional cytoprotective activities at least including anti-oxidative and anti-inflammatory activities. Thus, understanding the cellular protective mechanism of methyleugenol would provide a new drug discovery and development for the treatment of ischemic brain injury.

It is well known that ischemia and particularly post-ischemic reperfusion enhance the production of ROS in brain tissues [30]. Excessive production of ROS causes cell damage either directly by interacting and destroying cellular proteins, lipids and DNA, or indirectly by affecting normal cellular signalling pathways and gene regulation [31]. A number of previous studies have shown that treatment with antioxidants can reduce tissue damage following ischemic injury [30]. In the present study, we found that methyleugenol largely reduced the level of oxidative stress in *in vivo* as well as *in vitro* ischemia models. Thus, the levels of ROS determined by using HEt and DCF-DA were

significantly decreased by methyleugenol. DPPH reduction assay showed that methyleugenol has a direct, but weak, ROS scavenging activity, which is comparable to vitamin C. Our preliminary experiments and other researchers' studies [32] showed that ascorbic acid protects neurons from OGD/re-oxygenation toxicity due to its antioxidant activity. Thus, the anti-ischemic activity of methyleugenol may be attributed at least in part to the direct ROS scavenging activity shown in this study. In addition, methyleugenol largely up-regulated the activities of antioxidant enzymes including Mn-SOD and catalase. Taken together, therefore, reduced ROS levels in OGD/re-oxygenation-treated or immunostimulated glial cells could be due to direct ROS scavenging activity and antioxidant enzymes up-regulation by methyleugenol.

Substantial data suggest that nitric oxide (NO) is closely associated with ischemic brain injury. During the cerebral ischemic insult, iNOS expression is up-regulated in activated glial cells and peripheral leukocytes infiltrating into the ischemic brain lesion. Activated glial cells and leukocytes simultaneously produce NO and $O_2^{\cdot -}$ via expression and activation of iNOS [33,34]. NO and $O_2^{\cdot -}$ rapidly react in a 1:1 stoichiometry to form a strong oxidant $ONOO^-$ [35]. NO and its congeners are known to inhibit the mitochondrial respiratory chain, resulting in a loss of ATP and eventually leading to irreversible cellular damage [36]. Our previous studies showed that immunostimulated glial cells become very vulnerable to glucose deprivation due to over-expression of iNOS [37,38]. In iNOS knockout mice, infarct expansion after ischemia does not occur [39,40]. Further, ischemia-induced brain damage was prevented by aminoguanidine, a selective iNOS inhibitor [41]. These findings strongly suggest that NO contributes to delayed neurotoxicity in ischemic insults [36]. Thus, the protective effect of methyleugenol may be at least in part due to the decreased NO production via down-regulation of iNOS activity in activated glial cells.

Recently, much evidence has been accumulated to show that inflammatory responses play a critical role in continuous delayed post-ischemic injury [42]. We previously reported that MCAO/reperfusion increased the number of amoeboid cells in the entire ipsilateral hemisphere including cortex and striatum [43]. Most of those amoeboid cells are thought to be monocytes recruited from the periphery and some activated microglia to be morphologically transformed into amoeboid form. Furthermore, microglia rapidly respond to ischemic injury by secreting inflammatory cytokines [44]. Although cytokines are also produced by other cells including astrocytes, neurons and endothelial cells, microglia are most rapidly and highly activated in ischemic insult [45]. Our present findings that methyleugenol reduces the production of pro-inflammatory cytokines in ischemia/reperfusion-treated rat brain or immunostimulated mixed glial cells may in part explain how methyleugenol reduces the cerebral ischemic injury.

In summary, methyleugenol can protect neuronal cells from ischemic insults at least in part due to its anti-oxidant and anti-inflammatory activities. Further medicinal chemistry-based derivation and pharmacological study of methyleugenol would provide therapeutically useful drugs for the treatment of ischemia/inflammation-associated neurodegenerative diseases.

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