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Neuroprotective effects of PMC, a potent α-tocopherol derivative, in brain ischemia-reperfusion: Reduced neutrophil activation and anti-oxidant actions

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ABSTRACT

2,2,5,7,8-Pentamethyl-6-hydroxychromane (PMC) is the most potent analogue of α-tocopherol for anti-oxidation. It is more hydrophilic than other α-tocopherol derivatives and has potent free radical-scavenging activity. In the present study, PMC significantly attenuated middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia in rats. Administration of PMC at 20 mg/kg, showed marked reductions in infarct size compared with that of control rats. MCAO-induced focal cerebral ischemia was associated with increases in HIF-1α, active caspase-3, iNOS, and nitrotyrosine expressions in ischemic regions. These expressions were markedly inhibited by treatment with PMC (20 mg/kg). In addition, PMC (4–12 μM) inhibited respiratory bursts in human neutrophils stimulated by fMLP (800 nM) and PMA (320 nM). Furthermore, PMC (6, 12, and 60 μM) also significantly inhibited neutrophil migration stimulated by leukotriene B_4 (160 nM). An electron spin resonance (ESR) method was conducted on the scavenging activity of PMC on the free radicals formed. PMC (12 μM) greatly reduced the ESR signal intensities of superoxide anion, hydroxyl radical, and methyl radical formation. In conclusion, we demonstrate a potent neuroprotective effect of PMC on MCAO-induced focal cerebral ischemia in vivo. This effect may be mediated, at least in part, by inhibition of free radical formation, followed by inhibition of HIF-1α activation, apoptosis formation (active caspase-3), neutrophil activation, and inflammatory responses (i.e., iNOS and nitrotyrosine expressions), resulting in a reduction in the infarct volume in ischemia-reperfusion brain injury. Thus, PMC treatment may represent a novel approach to lowering the risk or improving function in ischemia-reperfusion brain injury-related disorders.

1. 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 and free radicals, platelet activation, and apoptosis [1,2]. Reperfusion of ischemic areas can exacerbate ischemic brain damage through the generation of reactive oxygen species (ROS)

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Abbreviations: ESR, electron spin resonance; fMLP, formyl-Met-Leu-Phe; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; LCL, lucigenin-enhanced chemiluminescence; MCAO, middle cerebral artery occlusion; ROS, reactive oxygen species; mAb, monoclonal antibody

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including superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals, and peroxynitrite radicals from activated neutrophils [3] and by excessive production of nitric oxide (NO) through the induction of inducible nitric oxide synthase (iNOS) [4]. Neutrophils 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 neutrophils to endothelial cells and generation of ROS that enhances neutrophil infiltration and results in ischemic injury [6]. It has been reported that NO immediately reacts with superoxide to form peroxynitrite ($ONOO^-$), which is capable of nitrating tyrosine residues of proteins and enzymes to generate nitrotyrosine leading to tissue injury [7]. Therefore, both inhibition of neutrophil activation and enhanced degradation of ROS with pharmacological agents have been found to limit the extent of brain damage following stroke-like events [6,8].

Furthermore, ROS also mediate a mitochondrial signaling pathway that may lead to apoptosis following cerebral ischemia [9]. Various in vitro studies have demonstrated that cellular or biochemical signaling pathways involve mitochrondria-derived activator of caspases, the activation of downstream caspase-9 and -3, and DNA fragmentation [9].

PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane), in which the phytyl chain is replaced by a methyl group (Fig. 1), is the most potent derivative of α-tocopherols in anti-oxidation and inhibits activation of NF-κB [10,11]. It is more hydrophilic than other α-tocopherol derivatives, and has potent free radical-scavenging activity [11]. Recently, we also found that PMC (20–50 μM) markedly inhibited the expression of iNOS in macrophages in vitro and reversed the delayed hypotension in rats with endotoxic shock [12].

By considering the pivotal roles of ROS, inflammatory responses, and apoptosis in ischemia-reperfusion-induced brain injury, the present study was designed to examine the mechanisms responsible for mediating PMC's neuroprotective effects using an MCAO-reperfusion model in rats. We used these findings to characterize the relationship between the inhibition of neutrophil activation and free radical formation in vitro as well as cerebroprotection afforded by PMC.

2. Materials and methods

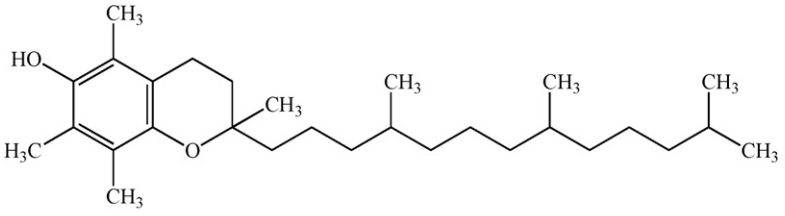
2.1. Materials

2,3,5-Triphenyltetrazolium (TTC), aprotinin, cremophor EL, leupeptin, lucigenin, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), *N*-formyl-Met-Leu-Phe (fMLP), phorbol 12-myristate-13-acetate (PMA), leukotriene B_4 (LTB_4), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). Ficoll-Paque plus was purchased from Amersham (Buckinghamshire, HP, UK). PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane), purchased from Wako Pure Chemical (Osaka, Japan), was dissolved in solvent (cremophor:ethanol:-normal saline 1:1:4) for the in vivo studies, and dissolved in 0.5% DMSO for the in vitro studies.

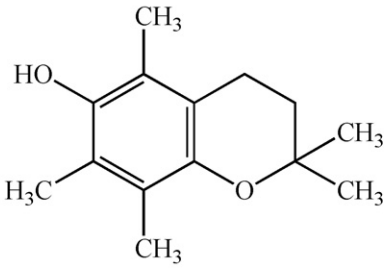
2.2. MCAO-induced transient focal cerebral ischemia in rats

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 clinically normal, were free of apparent infection or inflammation, and showed no neurological deficits.

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



α-tocopherol



PMC

Fig. 1 – Chemical structures of α-tocopherol and PMC.

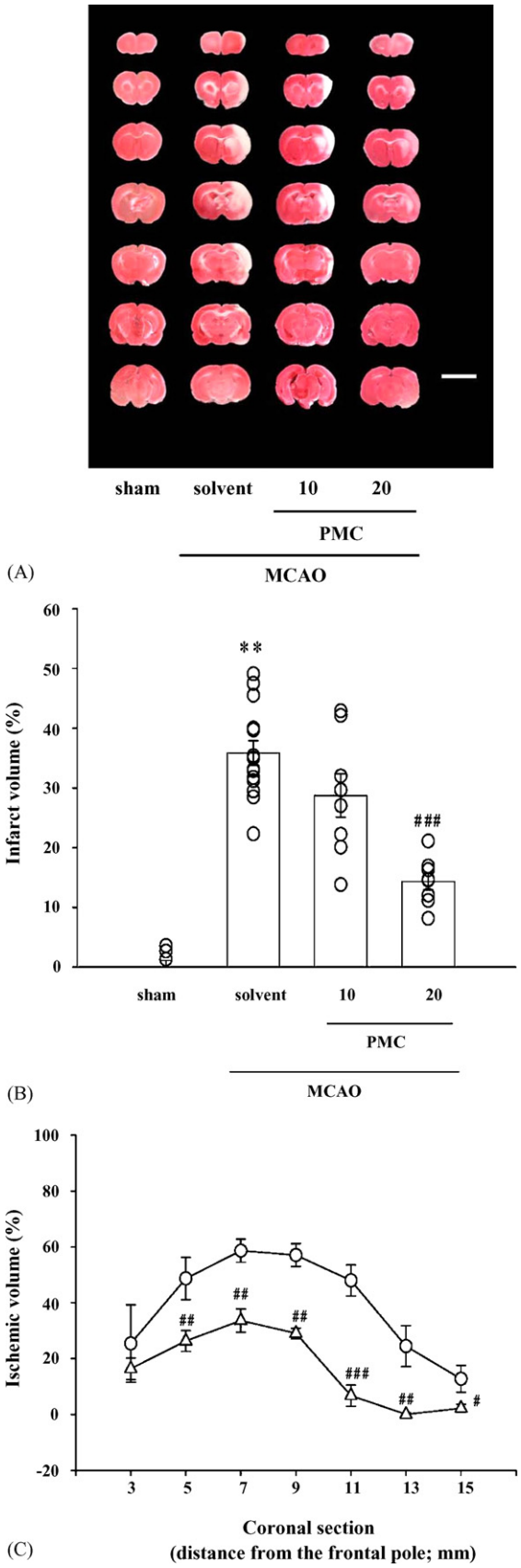


Fig. 2 – Effect of PMC in middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia in rats. (A) Coronal sections of TTC-stained brains, (B) dose-response effect of PMC, and (C) infarct area at various distances from the frontal pole in 24 h after MACO-reperfusion rats. Cerebral infarction in sham-operated (sham, *n* = 3) or MACO-reperfusion rats is from

(MCA) was occluded as described in our previous report [13]. Briefly, the right common carotid artery was exposed, and a 4–0 monofilament nylon thread (25 mm) coated with silicon was then inserted from the external into 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 the forelimb akinesia (also called postural tail-hang) test, whereas the spontaneous rotational test was used as a criterion for evaluating the ischemic insult [14]. 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 ipsilateral local blood flow to at least 60% of the baseline following an initial sharp decrease to about 30% of the baseline caused by MCAO as determined using a continuous laser Doppler flowmeter (Oxford Array™, Oxford Optronix, Oxford, UK) 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 (2% 2,3,5-triphenyltetrazolium, TTC) slice was drawn using a computerized image analyzer (Image-Pro plus). The calculated infarction areas were then compiled to obtain the infarct volumes (mm^3) per brain. Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using the formula: (the area of the intact contralateral [left] hemisphere − the area of the intact region of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere [15].

All animals were divided into four groups: (1) a sham-operated group; (2) a solvent-treated (solvent) group (cremophor:ethanol:normal saline 1:1:4); and groups treated with a single dose of (3) 10 or (4) 20 mg/kg, i.p. of PMC. In the group treated with the solvent or PMC, rats were given isovolumetric solvent or PMC (10 or 20 mg/kg) 10 min before MCAO.

2.3. Determination of the expressions of HIF-1α, caspase-3, and iNOS protein in MCAO-insulted brains

The expressions of HIF-1α, caspase-3, and iNOS in the brain 24 h after MCAO-reperfusion injury were analyzed by Western blotting as described by Rodrigo et al. [16] with some modifications. The MCAO-insulted and sham-operated rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), 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 drainge. Fresh brains were removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third parts of the right and left hemispheres were separately collected, snap-frozen in liquid nitrogen, and stored at −70 °C. The frozen tissues were weighed and placed in ice-hold homogenate buffer (50 mM HEPES, 100 mM KCl, 10 mM $MgCl_2$, 10 mM NaH_2PO_4, 10 μg/ml aprotinin, 1 mM PMSF, and 10 μg/ml leupeptin; pH 7.4) at a ratio of 1 g tissue to 1 ml buffer. Each brain tissue sample was homogenized using a polytron homogenizer, then sonicated for 10 s three times at 4 °C. The sonicates were subjected to centrifugation (10,000 × *g*).

The supernatant (50 μg protein) was subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes (0.45 μm; Hybond-P; Amersham). After incubation in blocking buffer (10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20, and 5% dry-skim milk; pH 7.5) and being washed three times with TBST buffer (10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5), the blots were treated with an anti-HIF-1α polyclonal antibody (pAb, 1:1000; R&D, Minneapolis, CA), anti-active caspase-3 pAb (1:250; Biovision, Mountain View, CA), and anti-iNOS mAb (1:3000, BD Biosciences, San Jose, CA) or an anti-tubulin mAb (1:2000; Santa Cruz Biotech, Santa Cruz, CA) or an anti-actin mAb (1:7000; Sigma) in TBST buffer overnight. Blots were subsequently washed four times with TBST and incubated with secondary horseradish peroxidase-conjugated goat anti-mouse mAb (Amersham) for 1 h. The blots were then washed, and the immunoreactive protein was detected using film exposure with enhanced chemiluminescence detection reagents (ECL^+ system; Amersham). The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using Videodensitometry (Bio-1D Version 99 image software).

2.4. Immunofluorescent analysis of nitrotyrosine expression in brain sections

The formation of nitrotyrosine in the brain 24 h after MCAO-reperfusion injury was detected as described previously [17] with some modifications. Following brain reperfusion, each post-anesthetic rat was perfusion-fixed with a 4% paraformaldehyde solution. The brains were removed and dehydrated with 30% sucrose. Fixed brains were immediately frozen and embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN). Samples were coronally cut into serial 10 μm-thick sections for immunohistofluorescent staining. Non-specific binding sites were blocked with 2% BSA, then sections were incubated for 2 h with a primary anti-nitrotyrosine mAb (1:50; Cayman, Ann Arbor, MI). Sections were then incubated for 1 h with an FITC-conjugated

representative animals that received solvent (solvent; cremophor:ethanol:normal saline 1:1:4, *n* = 14) or PMC (10 mg/kg, *n* = 8; 20 mg/kg, *n* = 8) intraperitoneally. (B) Infarct volumes were calculated as described in Section 2, and data are presented as a superimposed scatterplot showing the infarct volume for each animal in the group as well as the means ± S.E.M. $^{**}P < 0.001$ compared with the sham-operated group; $^{\#\#\#}P < 0.001$ as compared with the solvent-treated group. (C) Forebrain profiles of the infarct area at various distances from the frontal pole as described in Section 2. Each point (○, solvent-treated group, *n* = 14; ◇, PMC 20 mg/kg-treated group, *n* = 8) and vertical bar represent the means ± S.E.M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ as compared with the solvent-treated group.

secondary antibody (1:100; Amersham). They were washed three times in PBS (pH 7.2) between each step. The area of the cortex in the brain sections was observed by using a Nikon TS100 fluorescence microscope equipped with an optical filter to select the fluorescence emission (green). Images were captured using a digital color CCD camera (*CoolSNAP-Pro cf*) and Image-Pro Plus software 4.5. (Media Cybernetics, Silver Spring, MD).

2.5. Determination of respiratory bursts in human neutrophils

Neutrophils were isolated by sedimentation through dextran and Ficoll/Hypaque gradient centrifugation from healthy individuals as previously described [18]. Superoxide anion production of neutrophils was measured by the method of lucigenin-enhanced chemiluminescence (LCL) as described by Hsiao et al. [18] with some modifications. Washed neutrophil suspensions (2×10^6 cells/ml) in modified Hank's balanced salt solution (HBSS) containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were dispensed into wells of a standard scintillation microplate. Before the assay, cells were preincubated with the solvent control (0.5% DMSO) or various concentrations of PMC (4, 6, and 12 μM). Then, 20 μl aliquots of lucigenin were added at a final concentration of 100 μM. The basal LCL was recorded for 1 min by a microplate luminometer (Orion®, Berthold, Germany) at 37 °C, and cells were immediately stimulated with fMLP (800 nM) or PMA (320 nM). The luminescent light was continuously recorded for 5 min. The chemiluminescent signal was represented as relative light units per second (RLU/s). The results of LCL intensity (as increments in the signal intensity) were determined by measuring basal and stimulator-induced peak values and calculating the difference between them.

2.6. Determination of neutrophil migration

The neutrophil chemotaxis assay was performed using a 24-well transwell system (Corning, NY) as previously described [19]. The chemoattractant, LTB_4 (160 nM), or free buffer (as the negative control) was placed in the wells of 24-well tissue culture plates. The transwell inserts (8 μm pore size, polycarbonate membrane) were filled with neutrophils in modified HBSS. Plates was incubated for 90 min at 37 °C in 5% CO_2. The number of neutrophils that migrated to the bottom of the wells was quantitated using an inverted microscope equipped with phase-contrast objectives. Total migrating neutrophils were determined by counting the total number of cells in four randomly selected 40× microscope fields (200 μm × 200 μm) and these were also captured by a digital image system (Image-Pro Plus Software 4.5; MediaCybernetics).

2.7. Electron spin resonance spectrometry

Electron spin resonance (ESR) spectra were recorded on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 3456 ± 50 G; power of 0.635 mW; a modulation frequency of 100 kHz; a frequency of 9.663 GHz; a modulation amplitude of 1 G; receiver gain of 6.3×10^{-4}; a time constant of 81.92 ms; a conversion time of 327.68 ms. The ESR spectrum was obtained in the H_2O_2/NaOH/DMSO system as described previously [20]. Briefly, 100 μl of DMSO and the same volumes of 25 mM NaOH and PMC (12 μM) were mixed in a test tube, followed by the addition of 10 μl 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 (PMC)/signal height (solvent control)] [20].

2.8. Statistical analysis

The experimental results are expressed as the means ± S.E.M. 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. The other experiments were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman–Keuls method. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of PMC on MCAO-induced focal cerebral ischemia in rats

All animals in this study showed similar physiological values (i.e., rectal temperature, 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 solvent- or PMC-treated groups. The cerebral infarction was examined using 2-mm-thick slices of the cerebrum of 24 h after MCAO-reperfused rats through TTC staining. Fig. 2A shows typical photographs of coronal sections of the sham-operated group, the solvent (cremophor:ethanol:normal saline 1:1:4)-treated, and PMC-treated groups (10 and 20 mg/kg) prior to the ischemic insult. Administration of PMC at 10 and 20 mg/kg, showed concentration-dependent reductions in infarct volume (white area) compared with the solvent-treated group (solvent, 35.8 ± 2.1%, $n = 14$ versus 10 mg/kg, 28.7 ± 3.6%, $n = 8$; 20 mg/kg, 14.4 ± 1.4%, $n = 8$) (Fig. 2B). On the other hand, treatment with the solvent did not significantly influence the infarct size compared with the MCAO group (without treatment with solvent or drugs) (data not shown). Fig. 2C are statistical results of the infarct areas of solvent- and PMC (20 mg/kg)-treated groups at various distances from the frontal pole. The area was largest in the third section (out of four sections) in two groups. Treatment with PMC (20 mg/kg) reduced the infarct area in all regions, especially in the three to five sections (Fig. 2C). In solvent-treated rats, approximately 58.6 ± 4.1% of the entire area was infracted in the third section, while PMC (20 mg/kg) ingestion reduced the area to 33.6 ± 4.1% in the third section (Fig. 2C).

3.2. *Effects of PMC on HIF-1α, caspase-3, and iNOS expressions in ischemic cerebral tissues*

Results of Western blotting of MCAO-insulted cerebral tissues are shown in Figs. 3 and 4. As shown in Fig. 3A, HIF-1α, detected as a major band of approximately 120 kDa in the ipsilateral hemisphere 24 h after MCAO-reperfusion injury (lane 2) was more pronounced than that of the levels obtained in the corresponding areas of the sham-operated group (lane 1) or in the contralateral hemisphere (lane 5). PMC (20 mg/kg) treatment significantly ($P < 0.05$) suppressed the levels of HIF-1α in the ipsilateral hemisphere (Fig. 3A, lane 3). In the Fig. 3B, negative immunostaining was obtained for active caspase-3 in the sham-operated group (lane 1). At 24 h after MCAO-reperfusion, strong staining of active caspase-3 (17 kDa) was observed in the ipsilateral hemisphere (lane 2) compared with

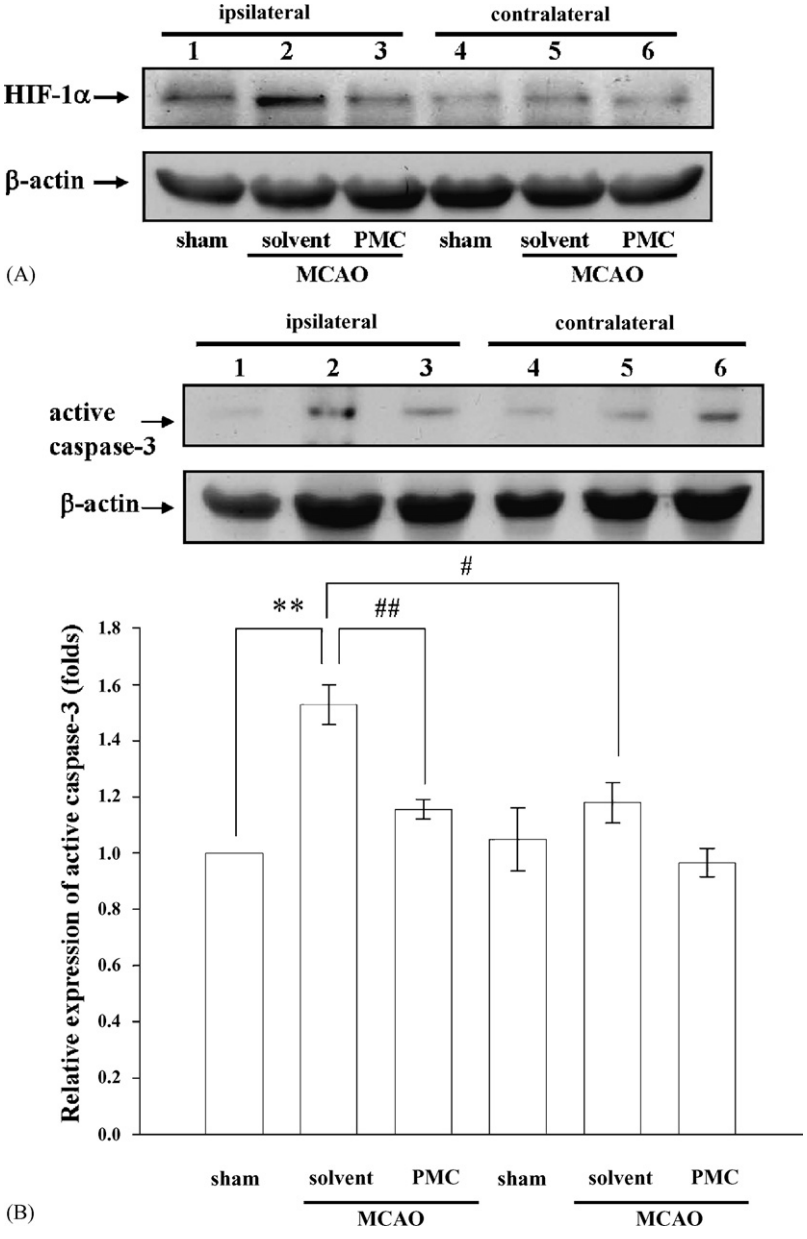


Fig. 3 – Effect of PMC on the expressions of (A) HIF-1α and (B) active caspase-3 in cerebral homogenates 24 h after middle cerebral artery occlusion (MCAO)-reperfusion injury in rats. Fresh brains from sham-operated (sham, lanes 1 and 4) or solvent-treated (solvent, lanes 2 and 5), and PMC (20 mg/kg)-treated (PMC, lanes 3 and 6) rats were removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third parts of the four sequential parts of the ipsilateral (right) and contralateral (left) hemispheres were separately collected, homogenized, and centrifuged. The supernatant (50 μg protein) was then subjected to SDS-PAGE, and transferred onto membranes for analysis of HIF-1α and active caspase-3 expressions. The results are representative examples of four similar experiments. Data are presented as the means ± S.E.M. $^{**}P < 0.001$ compared with the sham-operated group (lane 1); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with the solvent-treated groups (lane 2). Equal loading in each lane is demonstrated by similar intensities of α-actin.

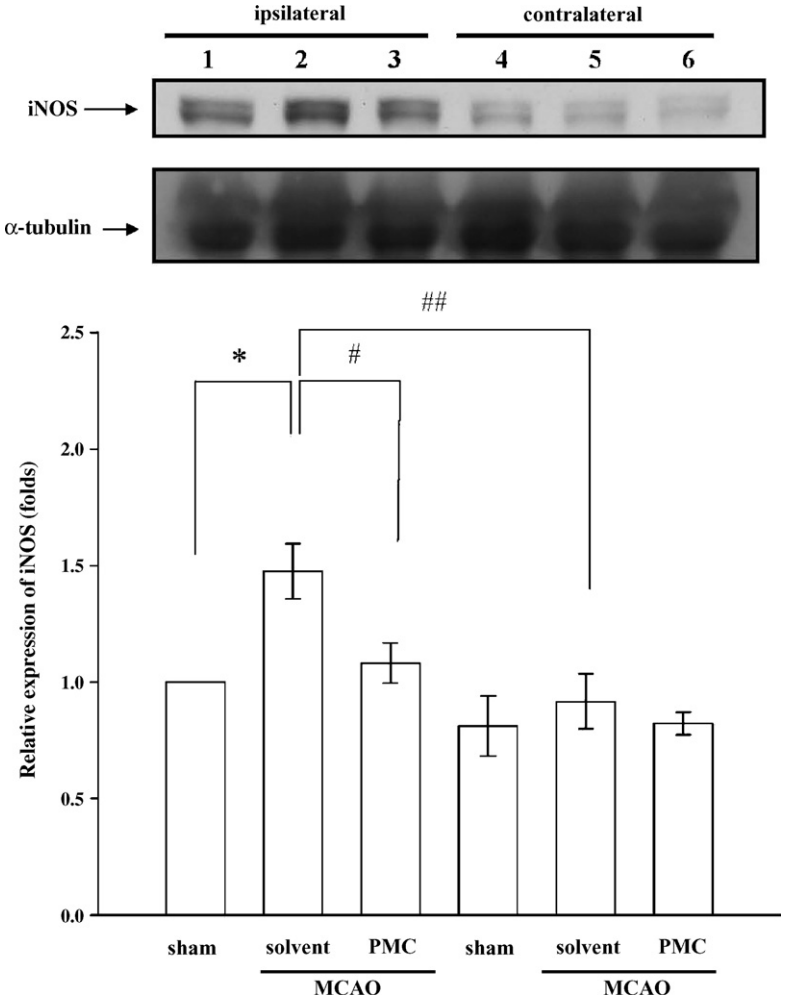


Fig. 4 – Effect of PMC on the expression of iNOS in cerebral homogenates after middle cerebral artery occlusion (MCAO)-reperfusion injury in rats. Fresh brains from sham-operated (sham, lanes 1 and 4) or solvent-treated (solvent, lanes 2 and 5), and PMC (20 mg/kg)-treated (PMC, lanes 3 and 6) rats were removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third parts out of the four sequential parts of the ipsilateral (right) and contralateral (left) hemispheres were separately collected, homogenized, and centrifuged. The supernatant (50 μg protein) was then subjected to SDS-PAGE, and transferred onto membranes for analysis of iNOS expression. The results are representative examples of seven similar experiments. Data are presented as the means ± S.E.M. $^{*}P < 0.01$ compared with the sham-operated group (lane 1); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with the solvent-treated group (lane 2). Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

the levels obtained in the corresponding areas of the sham-operated group (lane 1) ($P < 0.001$, $n = 4$) or in the contralateral hemisphere (lane 5) ($P < 0.01$, $n = 4$). Again, PMC (20 mg/kg) abolished the elevation of active caspase-3 ($P < 0.05$, $n = 4$) (Fig. 3B, lane 3).

In addition, the iNOS band, detected as a major band of approximately 135 kDa in the ipsilateral hemisphere (Fig. 4, lane 2), showed significant increases in ischemic cerebral tissues 24 h after MCAO-reperfusion as compared with that of sham-operated rats (Fig. 4, lane 1) ($P < 0.01$, $n = 7$) or in the contralateral hemisphere ($P < 0.01$, $n = 7$) (Fig. 4, lane 5). With administration of PMC (20 mg/kg), iNOS expression was markedly reduced in MCAO-reperfusion rats ($P < 0.05$, $n = 7$) (Fig. 4, lane 3).

3.3. *Effect of PMC on nitrotyrosine expression in ischemic cerebral regions*

The nitrotyrosine level was evaluated as a marker of peroxynitrite accumulation in the penumbra of brain ischemic regions. Peroxynitrite is generated by a combination of NO and superoxide in the ischemic penumbra [7]. This study revealed that the post-MCAO period was associated with an increase in nitrotyrosine expression in the cortex ischemic regions (Fig. 5B) compared with the corresponding areas of the sham-operated group (Fig. 5A) or in the contralateral cortex (Fig. 5E). The intensity of immunostaining was markedly attenuated in the cortex of ischemic regions in PMC (20 mg/kg)-treated rats (Fig. 5C). The intensity of staining was not

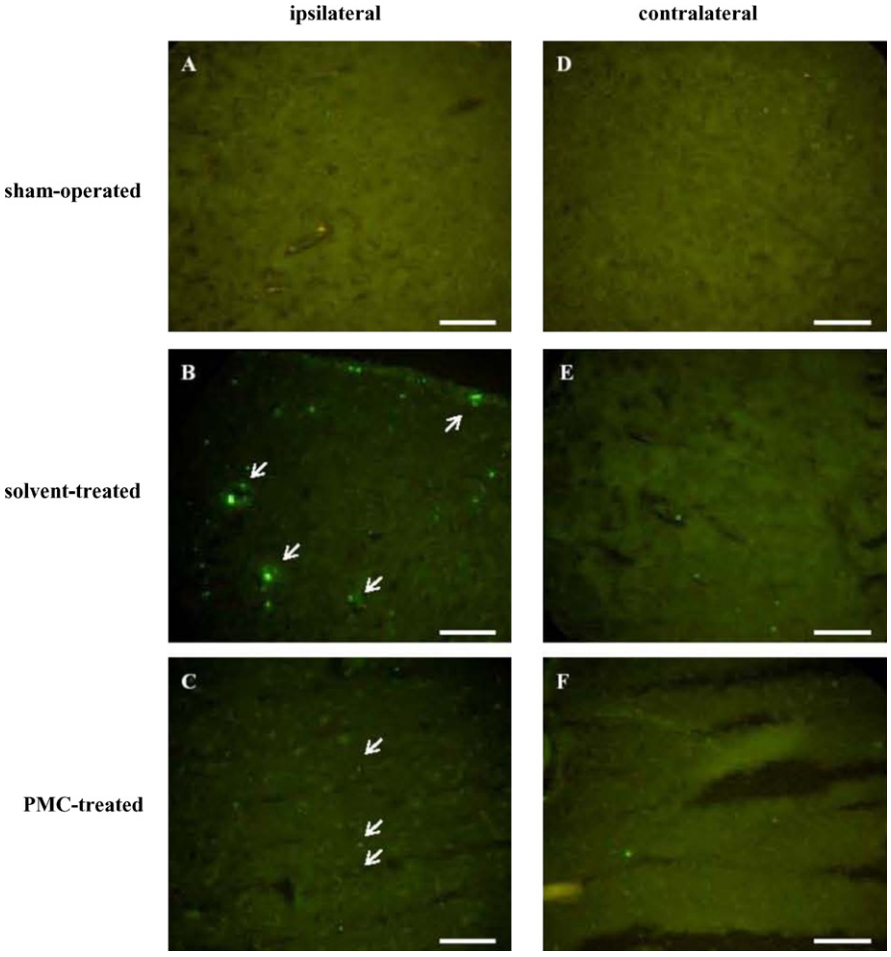


Fig. 5 – Effect of PMC on the expression of nitrotyrosine in the ipsilateral brain cortex after middle cerebral artery occlusion (MCAO)-reperfusion injury in rats. Immunofluorescent staining of nitrotyrosine in the ipsilateral and contralateral brain hemispheres (−0.3 mm from the bregma) 24 h after MCAO. A, B, and C are the ipsilateral cortex. D, E, and F are the contralateral cortex. A and D are the sham-operated group. B and E are the solvent-treated group. C and F are the group pretreated with PMC (20 mg/kg). Arrows indicate the expression of nitrotyrosine. The results are representative examples of four similar experiments (scale bar = 100 μm).

clearly seen in the areas of the contralateral cortex in sham-operated (Fig. 5D) or PMC (20 mg/kg)-treated groups (Fig. 5F).

3.4. *Effects of PMC on respiratory bursts and chemotaxis in human neutrophils*

The inhibitory effect of PMC on neutrophil activation was evaluated by fMLP- and PMA-induced lucigenin-dependent chemiluminescence (LCL), which is an index of respiratory bursts, as production of superoxide anions. When human neutrophils (2×10^6 cells/ml) were treated with fMLP (800 nM), a rapid generation of superoxide anions was observed with the LCL signal rising as high as 2775 ± 148 RLU/s ($n = 17$; data not shown). PMC (4–12 μM) concentration-dependently inhibited the increase in chemiluminescence stimulated by fMLP, with an IC_{50} of about 8.8 μM (Fig. 6). At 12 μM PMC greatly reduced the LCL stimulated by fMLP by about 79% as compared with the solvent control (0.5% DMSO). On the other hand, stimulation by PMA (320 nM) caused a gradual generation of superoxide anions from neutrophils, with a peak of LCL of as high as 447 ± 47 RLU/s ($n = 12$, data not shown). Similarly, the PMA-induced increase in chemiluminescence was attenuated by PMC (4–12 μM) in a concentration-dependent manner (Fig. 6). Furthermore, the superoxide anion production induced by these two stimulators was also markedly abrogated by superoxide dismutase (200 U/ml) (data not shown).

We further investigated the effect of PMC on the chemotaxis of neutrophils using the transwell method. In the presence of solvent (0.5% DMSO), human neutrophil migration increased to 40.2 ± 9.8 cells/field when stimulated by LTB_4 (160 nM) ($n = 5$, as the solvent control; data not shown) which was greater than that of spontaneously migrating cells at 5.2 ± 2.0 cells/field ($n = 5$; data not shown). Treatment of neutrophils with PMC (6, 12, and 60 μM) caused significant inhibition of LTB_4-induced chemotaxis in a concentration-dependent manner (Fig. 6). At a higher concentration (60 μM), the chemotactic response was attenuated to $51.7 \pm 7.7\%$ of the solvent control (Fig. 6).

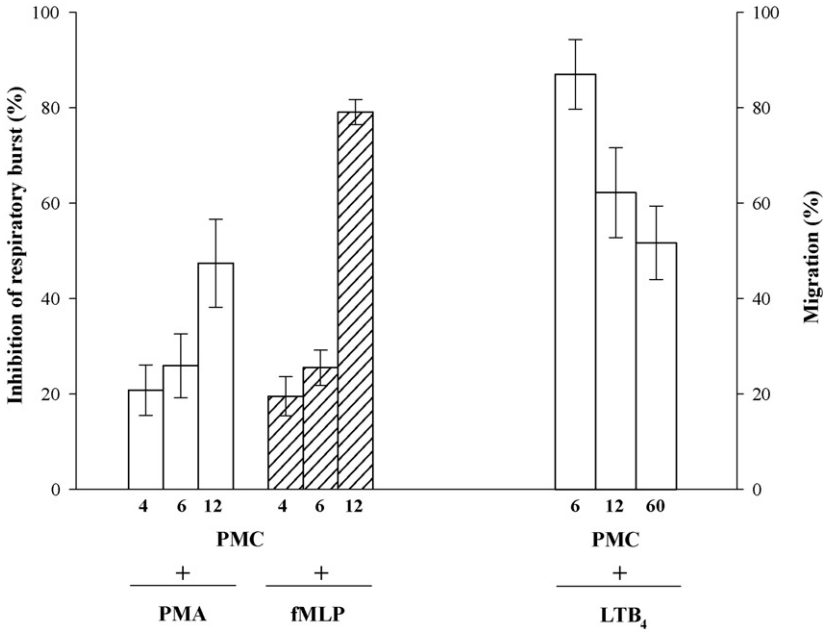


Fig. 6 – Effects of PMC on respiratory bursts and chemoattractant-induced cell migration in human neutrophils. Left panel, washed neutrophil suspensions (2×10^6 cells/ml) were preincubated with the solvent control (0.5% DMSO) or various concentrations of PMC (4, 6, and 12 μM) in the presence of lucigenin (100 μM), followed by the addition of PMA (320 nM) or fMLP (800 nM) to trigger neutrophil respiratory bursts. Data are presented as a percent inhibition of the solvent control (means ± S.E.M., $n = 4$). Right panel, neutrophil chemotaxis was evaluated using the 24-well transwell system. The chemoattractant of LTB_4 (160 nM) was placed in the 24-wells, and the transwell inserts were filled with neutrophils in the presence of the solvent control (0.5% DMSO) or various concentrations of PMC (6, 12, and 60 μM). The number of neutrophils that migrated to the bottom of the wells was quantitated using an inverted microscope and a digital image system. Data are presented as a percentage of the solvent control (means ± S.E.M., $n = 4$).

3.5. *Free radical-scavenging activity of PMC*

In this study, typical ESR signals of superoxide anions, hydroxyl radicals, and methyl radicals were observed as shown in Fig. 7. PMC (12 μM) markedly suppressed superoxide anion and hydroxyl radical formation by about 78 and 57% ($n = 4$), respectively (Fig. 7C). In addition, PMC (12 μM) also suppressed methyl radical formation by about 45% ($n = 4$); however, the suppression rate of PMC against methyl radicals was smaller than those against superoxide anions and hydroxyl radicals. This observation may further provide in vitro evidence suggesting the usefulness of PMC's free radical-scavenging activity.

4. Discussion

This study demonstrates for the first time that PMC possesses a neuroprotective effect after MCAO-reperfusion injury. 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 [21].

The present study demonstrated that MCAO-reperfusion injury induces increases in HIF-1α, active caspase-3, and iNOS protein expressions, and this may represent the response of neurons suffering from the ischemic insult. The increased HIF-1α protein levels observed after MCAO-reperfusion are presumably induced by a loss of the oxygen supply [22], resulting in a greater extent of binding activity to the iNOS gene and reaching a consequent peak of iNOS protein expression. Since the iNOS gene contains the hypoxia-responsive enhancer (HRE) sequence to which HIF-1α binds [23], 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 [24]. HIF-1α also regulates several target genes including glycolytic enzymes, erythropoietin and VEGF [25]. Furthermore, HIF-1α combines with p53 may promote apoptotic cell death in ischemic areas [26]. Furthermore, the increased expression of iNOS may also contribute to enhanced neuronal injury, since iNOS knock-out mice showed reduced brain damage after ischemia [27]. The toxic effects of NO may be attributed to peroxynitrite, which is a reaction product of NO with superoxide. Peroxynitrite is responsible for the nitration of both free and protein-bound tyrosine residues which are known to disrupt cell signaling cascades [28].

Several apoptosis-related proteins, including caspase-9 and -3 were all 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 Bcl-2 family proteins) [29]. Bax is translocated to the mitochondria where it releases cytochrome *c* into the cytosol to interact with Apaf-1 to

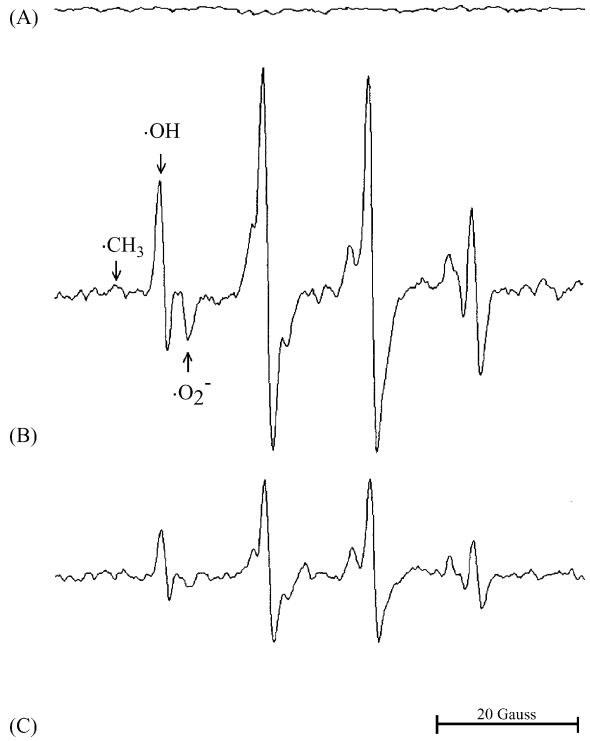


Fig. 7 – Effect of PMC on the free radical-scavenging activity in the H_2O_2/NaOH/DMSO system. ESR conditions are described in Section 2. Each free radical-derived signal was assigned, and signal heights are calculated in the figure. The intensity of the hydroxyl radical peak was much stronger (quartered signal peaks) than those of the superoxide anion and methyl radical peaks. (A) Resting spectrum (without H_2O_2); (B) typical ESR spectra in the presence of solvent control (0.5% DMSO) or (C) PMC (12 μM) in the H_2O_2/NaOH/DMSO system. The spectrum is a representative example of four similar experiments.

activate caspase-9, which in turn activates downstream caspases, such as active caspase-3 [30]. In the present study, we showed that elevations of active caspase-3 and iNOS expressions occurred in the same time frame as HIF-1α expression after ischemic injury, and these expressions can be significantly suppressed by pretreatment with PMC (20 mg/kg).

Leukocytes, particularly neutrophils, contribute to initiation of ischemic stroke. Thus, infiltration of neutrophils into the infarct areas following cerebral ischemia-reperfusion injury also plays a crucial role in the development of cerebral infarction and neuronal damage [6,31]. Although neutrophils produce and release a variety of toxic agents designed to kill microbes, those systems that depend on reactive products of oxygen metabolism are especially potent. These agents are produced as a consequence of respiratory burst, a series of events triggered by phagocytosis or exposure to certain inflammatory mediators and feature a dramatic increase in oxidative metabolism with direct conversion of molecular oxygen to its univalent reduction product, the superoxide anion. Subsequent reactions lead to the formation of other toxic species, including hydrogen peroxide, hydroxyl radicals, hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$) [32]. Reducing free radicals will reduce protein kinase activities and cytokine formation that derives the up regulation of inflammatory proteins like iNOS and apoptotic signaling like caspase-3. Our results showed that PMC significantly inhibited both neutrophil migration and respiratory bursts by different stimulators such as fMLP, PMA, and LTB_4, suggesting that PMC does not act at the level of a specific ligand–receptor interaction. Both the croton oil derivative, PMA, and the chemotactic tripeptide, fMLP, can activate leukocytes, resulting in leukocyte adhesion to the endothelium, leukocyte aggregation, decreased leukocyte deformability, and release of oxidants, proteases, and lipid metabolites [33]. LTB_4, the main product of neutrophil 5-lipoxygenase, is a powerful chemoattractant for neutrophils; however, it is a weak stimulator of neutrophil respiratory bursts [34]. In this study, the anti-migration effect of PMC was not due to its cytotoxic effect, because under these conditions, there was no significant difference in cell viability (>95% in all groups) between the PMC-treated group and the solvent-treated group (data not shown). Furthermore, we also examined whether PMC has direct free radical-scavenging activity in a cell-free system. In this study, the mechanisms of free radical formation in the H_2O_2/NaOH/DMSO system were assumed to be from superoxide anions and hydroxyl radicals being generated from the degradation of hydrogen peroxide, and methyl radicals being generated from the degradation of DMSO by hydroxyl radicals [20]. 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. Using this system, the free radical-scavenging activities of superoxide anions, hydroxyl radicals, and methyl radicals could simultaneously be evaluated. PMC effectively inhibited hydroxyl radical, superoxide anion, and methyl radical formation in vitro. Thus, the neuroprotective mechanisms of PMC may involve, at least partly, the inhibition of free radical formation.

On the other hand, the accumulation of ROS directly injures tissues through macromolecular damage (i.e., damage to DNA, lipids, proteins, etc.) [9]. In addition, ROS provides a redox signal for hypoxic HIF-1α activation [35]. In some cell types, increased ROS production is associated with HIF-1α activation by hormones, growth factors, and transition metals [35,36]. ROS are required for the post-transcriptional stabilization of HIF-1α during hypoxic incubation of cells [36]. Therefore, the application of anti-oxidants may eliminate the hypoxic generation of ROS and concurrently reduces HIF-1α levels by destabilization of the protein, resulting in the loss of transcriptional activity [37]. PMC is a potent anti-oxidant that is approximately 9-, 18-, and 68-times more potent than butylated hydroxy toluence (BHT), α-tocopherol, and trolox in inhibiting lipid peroxidation [11]. The neuroprotective effect of α-tocopherol has been reported to correlate with a decrease in brain glutamate concentration and an increase in brain ATP after MCAO [38]. In the present study, we further demonstrate that PMC markedly suppressed HIF-1α expression, which may be mediated, at least partly, by inhibition of ROS formation.

In conclusion, the most important findings of this study suggest that the neuroprotective effect of PMC on cerebral ischemic damage in MCAO-reperfusion rats is assumed to be

mediated by the inhibition of ROS (free radical) formation, followed by inhibition of HIF-1α activation, apoptosis formation (active caspase-3), neutrophil activation, and inflammatory responses (i.e., iNOS and nitrotyrosine expressions). The rationale for the use of PMC 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 PMC is not limited to one factor but involves many mechanisms, most of which may be interrelated. For example, PMC-induced neuroprotection is related to free radicals, inflammation, NO, and apoptosis, and many of those factors are related to HIF-1α (such as iNOS, active caspase-3, etc.). We speculate that the correction of these molecules and morphological changes may lead to neurobehavioral improvement in patients, and treatment with PMC may represent a novel approach for improving function after ischemia-reperfusion brain injury.

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