Vanillin Attenuates Pro-Inflammatory Factors in tMCAO Mice Model Via Inhibiting of TLR4/NF- κ B Signal Pathway

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Research

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Abstract

Purpose

Vanillin has been reported to reduce hippocampal neuronal death in rats of global cerebral ischemia. However, the immunoregulatory mechanism of vanillin in ischemic mice is still unclear. Hence, this study aims to investigate the role of vanillin in transient middle cerebral artery occlusion (tMCAO) mice.

Methods

Transient cerebral ischemic stroke was induced by tMCAO surgery following by reperfusion in mice. After 24 hours of ischemia/reperfusion, Bederson scoring and TTC staining were used to evaluate neurological deficit and infarct volume, respectively. Furthermore, the expression of cytokines in ipsilateral hemisphere were detected by qPCR, ELISA and immunofluorescence. In vitro, LPS-stimulated primary and BV2 microglia cells were used to mimic neuroinflammation after ischemic stroke. Similarly, the expression of cytokines was detected by qPCR and ELISA. In addition, Western blotting was performed to evaluate the expression of Toll-like receptor 4 (TLR4), nuclear factor- κ -gene binding p65 (NF- κ B p65) and phosphorylated NF- κ B p65.

Results

Vanillin reduced infarct volume and improved motor function after ischemia/reperfusion. IL-1 β and TNF- α were decreased in ischemic brain tissue of tMCAO mice after vanillin treatment. Similar changes were confirmed using the in vitro LPS-stimulated microglia cell model. Moreover, the decreasing expression of pro-inflammatory cytokines in vanillin group were related to TLR4/NF- κ B signal pathway.

Conclusions

Taken together, vanillin decreased activation of microglia by inhibiting TLR4 /NF- κ B signal pathway, and then reduced expression of pro-inflammatory cytokines IL-1 β and TNF- α , which finally reduced infarct volume and improve motor function in tMCAO mice.

Introduction

Stroke is the second leading cause of death in the world, with the characteristics of high incidence, high recurrence, high disability and high mortality. It is reported that 80% stroke are ischemic stroke[1], which is caused by vascular occlusion that blood cannot flow into brain and results in tissue damage. At present, recombinant tissue-type plasminogen activator (rt-PA) is the only recognized effective therapy for ischemic stroke[2]. However, due to technical requirements, many patients do not benefit from rt-PA treatment. In recent decades, drugs and methods for the treatment of ischemic stroke have mainly

focused on neuroprotection. However, few drugs have been translated into clinical trials[3]. Therefore, there is an urgent need to investigate new targets and drugs for the treatment of ischemic stroke.

Neuroinflammation is considered to be an important factor affecting the prognosis of ischemic stroke, so intervention for inflammatory response has become research hotspot. In the acute phase of ischemic stroke, microglia play neuroprotective or neurotoxic effects[4]. After ischemic stroke, microglia migrate to the lesion site and aggravate tissue damage by producing inflammatory cytokines and cytotoxic substances. On the other hand, microglia also promote tissue repair and remodeling by removing cell debris and producing anti-inflammatory cytokines[5]. Hence, therapies that inhibit the activation of pro-inflammatory microglia while augment repair would offer great promise for ischemic stroke. Nevertheless, during acute phase, TLRs is considered to play a key role after ischemic stroke and thus is an important therapeutic target[6]. Above all TLRs, TLR4 is mainly expressed on microglia and participates in the regulation of microglia activation after ischemic stroke[7]. It was reported that TLR4 is one of the targets of neuronal injury and inflammation, and the regulation of TLR4/MyD88/NF- κ B signal pathway is expected to improve the prognosis of ischemic stroke[8],[9].

Vanillin (4-oxy-3-methoxybenzaldehyde, C₈H₈O₃) is a small molecular obtained from orchid pods. It was reported that vanillin has a variety of biological activities, including anti-mutagenesis, antibacterial, anti-tumor, anti-oxidation, anti-inflammation and neuroprotection. For example, vanillin has been shown to reduce hippocampal neuronal death caused by global cerebral ischemia[10]. Recently, it has been found that vanillin could pass through the blood-brain barrier (BBB), decreasing BBB damage and oxidative damage, and reducing infarct volume as well as brain edema after hypoxic-ischemic brain injury[11], which suggested that vanillin may be an effective drug for the treatment of ischemic stroke. Nevertheless, the role of vanillin in ischemic stroke has not been investigated to date. Here, our study illustrated the neuroprotective effect of vanillin on regulating the expression of inflammatory cytokines in tMCAO mice, and the suppression of TLR4/NF- κ B signal pathway was involved in the anti-inflammatory mechanism of vanillin.

Methods

Cell Culture

The immortalized microglia BV2 cell line was purchased from China Center for Type Culture Collection and the test for mycoplasma contamination was negative. Cells were cultured in DMEM medium with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA, 10099141C) in a 5 % CO₂ incubator at 37 °C. In vitro isolation and culture of primary microglia, cells were isolated from newborn Sprague-Dawley rat as previously described[12]. By utilizing anti-Iba1 antibodies (Millipore, MA, USA, MAB360, 1:300) for immunofluorescence staining, we verified the purity of the primary microglia cells was more than 95%. We used LPS-stimulated microglia cells to mimic the activated microglia after stroke. Vanillin was obtained from Sigma-Aldrich (St. Louis, MO, USA, V1104).

tMCAO

Young adult C57BL/6 male mice (22g-26g) were purchased from Guangzhou University of Chinese Medicine, with free access to food and water on light cycle 12/12 h light/dark. All procedures were in accordance with the Animal Use and Care Committee for Research and The Fifth affiliated Hospital of Sun Yat-sen University (No. 00081). All animals were randomly divided into groups according to their body weight before operation, and fasted for 12 hours. The mice were anesthetized with isoflurane and transient cerebral ischemia was induced by inserting a nylon monofilament into middle cerebral artery. Body temperature was maintained at 37°C through a feedback heating pad. tMCAO was induced by 90 min of reversible middle cerebral artery occlusion following by 24 hours reperfusion. Sham-operated animals only underwent the procedure of separating artery, but the nylon monofilament was not advanced into the artery. Animals' exclusion criteria: death, subarachnoid hemorrhage, Berderson scoring less than 1 point or more than 4 points. During the whole procedure, investigators were blinded with the treatment group.

Groups and Berderson scoring

C57BL/6 mice were randomly divided into 5 groups. Different treatments were given after 90 min occlusion according to the grouping and body weight by tail vein injection. 1. Sham operation group (n=3). 2. tMCAO group (n=6): animals were given normal saline (8ml/kg) after 90 minutes of ischemia. 3. Low concentration (7mg/kg) of vanillin group (n=6): animals were given vanillin (7mg/kg) after 90 minutes of ischemia. 4. Medium concentration (28mg/kg) of vanillin group (n=6): animals were given vanillin (28mg/kg) after 90 minutes of ischemia. 5. High concentration (56mg/kg) of vanillin group (n=6): animals were given vanillin (56mg/kg) after 90 minutes of ischemia. Berderson scoring was performed after 24 hours reperfusion to evaluate neurological deficit as following: score 0: no obvious neurological deficit symptoms; 1: the left forelimb buckling to the chest wall, the right forelimbs extending to the ground when suspending; 2: failure to overcome the resistance; 3: rotating spontaneously towards the left; 4: no spontaneous walking; 5: animal death. The mice with a score of 1-3 were included in the experiment.

Quantitative evaluation of infarct volume

2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, USA, T8877) staining was used to evaluate infarct volume. Following by 90 min ischemia and 24 hours reperfusion, mice were anesthetized deeply with isoflurane. The brain was rapidly removed and frozen at -20°C for 25 min. Then brain tissues were sliced coronally into 2-mm thick sections. The sections were stained with 1.0% TTC at 37°C for 15 min and fixed in a 4.0% paraformaldehyde solution at room temperature for 12 hours. Finally, the brain sections were photographed by a digital camera and analyzed using Adobe Photoshop CS6. The infarct volume was calculated as following: corrected infarct volume = contralateral brain volume - (ipsilateral brain volume - infarct volume).

Real-Time qPCR

Total RNA was obtained using Trizol kit (EZBioscience, USA, B0004D) according to the manufacturer's procedure. Then cDNA was generated with 1 µg of total RNA. After that, SuperReal qPCR PreMix (SYBR Green) (Tiangen, Beijing, China, FP202-01) was used to perform the RT-qPCR reaction on a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's procedure. Samples were subjected to 40 cycles of amplification at 95 °C for 15s and 60 °C for 60s. IL-1β: F 5'- CTTTGAAGAAGAGCCCGTCC-3', R: 5'- GAGCTTTCAGCTCACATGGG-3'; TNF-α: F 5'- GACACCCCTGAGGGAGCTGA -3', R: 5'- CTCCAAAGTAGACCTGCCCCG -3'; IL-4: F 5'- CGAGATGTTTGTACCAGACG -3', R: 5'- GAACCCAGACTTGTTCCTTC -3'; IL-10: F 5'- CAACATACTGCTGACAGATT -3', R: 5'- CTGGGCCATGGTTCTCTGCC -3'; GAPDH: F 5'- TGGGGCCAAAAGGGTCATCA -3', R: 5'- GCAGGATGCATTGCTGACAA -3'.

Cytokine Analysis by ELISA

Secretion levels of IL-1β, TNF-α, IL-4 and IL-10 in each group were detected by ELISA (RayBio, Atlanta, USA). The brain tissues were grinded with 500 µ L PBS with 1% protease inhibitor. The tissue or cell samples were performed by according to the manufacturer's instructions. Three replicates of each sample were analyzed in each assay. Assays were repeated on three separate occasions.

Immunofluorescence

After 24 hours of ischemia/reperfusion, the brains were perfused with PBS, fixed with paraformaldehyde overnight, and embedded with paraffin. Next, the brains were coronally sliced into 10 µ m thick sections. The slices were dipped into xylene and series of ethanol gradient in order to dewax and rehydrate. The brain slices or fixed cell culture were blocked by 10% horse serum albumin (Biosharp, China, BL209A) and then incubated with anti-IL-1β antibody (RD, Minnesota, USA, AF-401), anti-TNF-α antibody (CST, Massachusetts, USA, 11948), anti-IL-4 antibody (Thermo Fisher Scientific, Waltham, MA, USA, PA5-115416), anti-IL-10 antibody (Abcam, Cambridge, UK, ab33471) and anti-Iba1 antibody (Millipore, MA, USA, MAB360, 1:300) overnight at 4°C. These were followed by incubation with corresponding secondary antibodies and then the nuclei were stained with Hoechst (Solarbio, China, B8040) at room temperature. Images were captured with a Nikon A1 spectral confocal microscope.

Western blotting

The levels of TLR4, NF-κB p65, and phosphorylated NF-κB p65 expression were measured by Western blot. Total proteins were lysed in M-PERTM Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA, 78503) containing with protease inhibitor cocktail (Millipore Corporation, Bedford, MA, USA, 539131-10 VL). Protein concentrations of each sample were qualified by using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA, 23225). Protein samples (30 µg per lane) were separated on 12% SDS-PAGE and then transblotted onto PVDF membranes (Thermo Fisher Scientific, Waltham, MA, USA, 88520). Blots were blocked with 5% skim milk for 1 hour and then were incubated with the primary antibodies against TLR4 (SAB, Maryland, USA, 35463), NF-κB p65 (SAB, Maryland, USA, 11014), phosphorylated NF-κB p65 (SAB, Maryland, USA, 21014) and GAPDH. After 12

hours, the membranes were incubated with corresponding secondary antibodies for 1 hour at room temperature. Finally, the blot images were detected with enhanced chemiluminescence reagent (Millipore, MA, USA, WBKLS0500) and captured using ChemiDoc™ MP System (Bio-Rad, UK).

Statistical analysis

GraphPad Prism 6.0 and IBM SPSS Statistics 25 were used to analyze the experimental data. All data were given as the mean $\pm$ SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc comparisons LSD test for multiple comparisons. P-values less than 0.05 was considered as statistically significant.

Results

Vanillin reduced infarct volume and promoted motor function in tMCAO mice

As showed in Fig. 1a, 1b and Additional file 1, compared with sham-operated mice, 90 min of ischemia following by 24 hours reperfusion resulted in 36.3 ± 6.5 infarct volume in whole brain. Low dose concentration of vanillin (7 mg / kg) did not reduce infarct volume in tMCAO mice. 28 mg / kg concentration of vanillin could decrease infarct volume but the result without statistical difference. Nevertheless, high dose concentration of vanillin (56 mg / kg) significantly decreased infarct volume ($p = 0.017$) to 23.3 ± 9 . In addition, compared with saline group, high dose concentration of vanillin ameliorated motor function deficit in tMCAO mice (Fig. 1c). Therefore, in following animal in vivo research, 56 mg / kg concentration of vanillin was used.

Vanillin reduced the expression of pro-inflammatory cytokines of IL-1 β and TNF- α in ipsilateral hemisphere

It was reported vanillin has multiple anti-inflammatory effect. To investigate whether vanillin regulated cytokines expression, qRT-PCR and ELISA were used to detect the expression level of pro-inflammatory cytokines (such as IL-1 β and TNF- α) and anti-inflammatory cytokines (such as IL-4 and IL-10) in ipsilateral hemisphere. As shown in Fig. 2a-d, mRNA levels of IL-1 β , TNF- α and IL-4 increased to 8.5 ± 0.7 , 3.2 ± 0.5 , 2.6 ± 0.4 folds in ipsilateral hemisphere, respectively, compared with sham-operated mice. Furthermore, protein levels of IL-1 β , TNF- α and IL-4 in ipsilateral hemisphere increased (Fig. 2e-h). Vanillin significantly reduced the mRNA level of pro-inflammatory cytokines, IL-1 β and TNF α , to 5.9 ± 0.6 , 1.9 ± 0.7 folds respectively. Similarly, vanillin significantly decreased the protein levels of pro-inflammatory cytokines, IL-1 β and TNF- α , to 69.9 ± 10.3 and 165.6 ± 15.1 folds respectively. However, vanillin did not regulate the expression levels of anti-inflammatory cytokines IL-4 and IL-10. Furthermore, we verify the expression levels of cytokines using immunofluorescence. In accordance with the result of ELISA, vanillin reduced the expression of IL-1 β and TNF- α (Fig. 2i-j), and the expression of IL-4 and IL-10 did not be influenced (see Additional file 2).

Vanillin inhibited expression of pro-inflammatory cytokines by LPS stimulated primary microglia

IL-1 β and TNF- α mainly originated from activated microglia or monocytes / macrophages in ischemic brain[13]. We further investigated whether vanillin regulated the expression of cytokines in LPS stimulated primary microglia. Immunofluorescence staining showed that the purity of isolated primary microglia was beyond 95% (Fig. 3a). Besides vanillin treatment, we used dexamethasone (Dexo) as a positive control, which was reported to inhibit microglia / macrophage activation stimulated by LPS in vitro. As shown in Fig. 3b-i, mRNA and protein levels of pro-inflammatory cytokines IL-1 β and TNF- α increased significantly ($P < 0.05$) but anti-inflammatory cytokines IL-4 and IL-10 did not be affected by LPS stimulation. Compared with LPS treatment group, vanillin decreased mRNA and protein levels of IL-1 β and TNF- α although mRNA level of TNF- α and secretion level of IL-1 β did not have statistically significant difference. As for dexamethasone group, it also decreased the mRNA and protein levels of IL-1 β and TNF- α in primary microglia stimulated by LPS. Furthermore, dexamethasone significantly increased expression of anti-inflammatory IL-4 in primary microglia cells.

Vanillin inhibited expression of pro-inflammatory cytokines by LPS stimulated BV2 cells

In order to further prove that vanillin reduced expression of pro-inflammatory cytokines in vitro, we also verified it in BV2 microglia cell. As shown in Fig. 4a-h, by using LPS stimulated, the mRNA and protein levels of IL-1 β and TNF- α significantly elevated in BV2 cells ($P < 0.05$). Vanillin significantly decreased mRNA levels of IL-1 β and TNF- α in LPS stimulated BV2 cells. Unlike primary microglia, the mRNA levels of IL-4 and IL-10 also elevated, and vanillin reversed these changes. However, the protein levels of IL-4 and IL-10 did not be regulated in vanillin treatment, compared with LPS group. Similar to primary microglia, dexamethasone not only significantly decreased mRNA and protein levels of IL-1 β and TNF- α , but also regulated the expression of IL-10.

Vanillin inhibited activation of microglia by inhibiting TLR4/NF- κ B signal pathway

It was reported that TLR4/NF- κ B signaling pathway plays an important role in microglia activation, which regulates the expression of inflammatory mediators[14],[15]. Hence, we investigated whether vanillin participated in the activation of microglia through TLR4/NF- κ B signaling pathway. We used LPS to stimulate BV2 cells or primary microglia, and 30 minutes later, cell protein samples were collected for Western blot. As shown in Fig. 5a-d, LPS significantly increased the expression of TLR4 as well as phosphorylated NF- κ B p56. Vanillin pretreatment significantly reduced the expression of TLR4 and decreased phosphorylated NF- κ B p65. These results suggested that vanillin may regulate pro-inflammatory cytokine expression in microglia by inhibiting TLR4/NF- κ B pathway.

Discussion

Vanillin is a flavouring agent, which has been widely used in the food, pharmaceutical and cosmetics industries. At present, more and more studies have shown that vanillin could improve neurological impairment, such as potassium bromate-induced neurotoxicity, LPS-induced motor dysfunction, rotenone induced in rat model of Parkinson's disease, neuroinflammation induced by β -amyloid and so on[16],[17],[18],[19]. It was reported that vanillin could cross the blood-brain barrier, making it possible to play its

neuroprotective and anti-inflammatory effects in CNS system. Furthermore, Xiaobing Lan et al. showed that vanillin reduces hypoxic ischemic brain damage and improved neurological function in neonatal rats[11]. Our study found that vanillin decreased infarct volume and improved motor function in tMCAO mice.

Cytokine is a kind of small molecule protein with extensive biological activity. A series of pro-inflammatory cytokines and anti-inflammatory cytokines involved in ischemic stroke. Pro-inflammatory factors were considered to relating in immune cell activation, tissue injury and necrosis, while anti-inflammatory factors inhibiting and ultimately reversing the inflammatory process[20]. IL-1 β and TNF- α were the widely studied pro-inflammatory cytokines in inflammatory response. Lambertsen KL revealed that IL-1 β and TNF- α increase in serum and CSF during acute ischemic stroke. It was also reported that both the mRNA expression of IL-1 β and TNF- α increase in damaged brain tissues of MCAO mice[21]. In accordance with those studies[22],[23], our results indicated mRNA and protein levels of IL-1 β and TNF- α increased in damaged brain of tMCAO mice. Furthermore, vanillin attenuated inflammation by decreasing expressions of IL-1 β and TNF- α in brain of tMCAO mice. Previous studies suggested that vanillin plays an anti-inflammatory role by suppressing the expression of proinflammatory cytokines. Compared with acute lung injury model group, vanillin inhibits expression levels of pro-inflammatory mediators TNF- α and IL-1 β in lung tissue[24]. Moreover, vanillin treatment decreases the protein levels of TNF- α and IL-1 β in mastitis mice compared with model group[25]. Similar to those researches, we demonstrated that vanillin decreased the expression of TNF- α and IL-1 β in tMCAO mice.

IL-4 and IL-10 are important anti-inflammatory cytokines which play a key role in promoting inflammation resolution and tissue repair. Xiong X found that the ischemic damage in brain is more severe in IL-4-deficient mice, but it can be reversed via intraventricular injection of IL-4[26]. IL-10 is a kind of multi-directional anti-inflammatory cytokine which inhibits the expression of TNF- α and IL-6 in monocytes / macrophages and inhibits the release of MMPs and ICAM-1[27]. It was reported that exogenous supplement of IL-10 significantly reduces infarct volume and IL-10 could decrease apoptosis and axonal injury after glucose deprivation in vitro[28],[29]. However, our results suggested that vanillin plays its therapeutic effect by selectively regulating the expression of pro-inflammatory cytokines without affecting anti-inflammatory cytokines.

IL-1 β and TNF- α mainly originated from activated microglia and monocytes / macrophages after ischemic stroke[13]. Hence, we wondered whether vanillin regulated the expression of cytokines via microglia. LPS-stimulated microglia have been widely used to investigate various inflammatory diseases such as ischemic stroke, multiple sclerosis and Alzheimer's disease[30]. Using LPS-stimulated primary microglia and BV2 microglia cells, our results indicated vanillin significantly decreased the expression of IL-1 β and TNF- α . This coincided with previous study, which suggested that vanillin reduces the mRNA and protein levels of IL-1 β and TNF- α in LPS- activated THP-1 cells[31]. However, our study further disclosed that vanillin did not regulate expression of anti-inflammatory cytokines in vitro. In addition, activated microglia can be divided into pro-inflammatory M1 type and anti-inflammatory M2 type according to

different state, function and secreted factors [32]. Whether the anti-inflammatory mechanism of vanillin involves the transformation between M1 and M2 needs to be further investigated.

TLR4/NF- κ B signaling pathway participates in inflammatory processes of many CNS diseases. Recent studies indicated that TLR4, MyD88 and NF- κ B may involve in pathophysiology after ischemic stroke[7]. Compared with other TLRs, TLR4 is more important in ischemic stroke[33]. TLR4 activates NF- κ B when it combines with its ligand such as LPS. Next, activated NF- κ B could translocate into nucleus and then regulate multiple inflammatory response, such as the expression of IL-1 β and TNF- α [34]. It was reported LPS-stimulated microglia involves in the activation of TLR4/NF- κ B signaling pathway, and inhibiting the activation of TLR4/NF- κ B pathway reduces the expression of inflammatory mediators in microglia[15]. Guo W indicated that vanillin reduces inflammation in mastitis mice by inhibiting TLR4/NF- κ B pathway[25]. Consistent with other studies, our findings showed that vanillin decreases the expression of TLR4 and phosphorylated NF- κ B p65, which indicated vanillin might decrease expression of IL-1 β and TNF- α in microglia via inhibiting TLR4/ NF- κ B signal pathway.

In conclusion, we found that vanillin decreased the activation of microglia by inhibiting TLR4 /NF- κ B signal pathway, and then reduced the expression of pro-inflammatory cytokines IL-1 β and TNF- α , and finally reduced infarct volume and improved motor function in tMCAO mice. Moreover, our study also found that vanillin played its anti-inflammatory effects without involving anti-inflammatory regulation, which hinted a promising drug for vanillin in the treatment of ischemic stroke.

Abbreviations

BBB: blood-brain barrier, tMCAO: transient middle cerebral artery occlusion, NF- κ B: nuclear factor- κ -gene binding, rt-PA: recombinant tissue-type plasminogen activator, TLR4: Toll-like receptor 4.

Declarations

Ethics approval: C57BL/6 mice were purchased from Guangzhou University of Chinese Medicine. All procedures were in accordance with the Animal Use and Care Committee for Research and The Fifth Affiliated Hospital of Sun Yat-sen University.

Consent for publication: Not applicable.

Availability of data and materials: The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Author Contributions: L-Z designed research, intellectual input, supervision; P-W and CY-L performed research, analyzed data, wrote the paper; GL-L, YH-H, XX-L and XD-L performed research, corrected the paper; WL-C: corrected the paper, provided directions for experimental design. L-Z, P-W, CY-L and WL-C contribute equal to this manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Vanillin reduced infarct volume and promoted motor function in tMCAO mice (a) TTC staining after 90 minutes of occlusion and 24 hours of reperfusion in tMCAO mice. (b) were the corrected infarct volume and (c) were the Berderson neurological function score in different group of tMCAO mice. n (Sham) = 3, n (tMCAO) = 7, n (7mg/kg) = 6, n (28mg/kg) = 6, n (56mg/kg) = 6. * $p < 0.05$, *** $p < 0.001$.

Figure 2

Vanillin reduced the expression of pro-inflammatory cytokines of IL-1β and TNF-α in ipsilateral hemisphere (a-d) were mRNA levels of IL-1β, TNF-α, IL-4 and IL-10 in ipsilateral hemisphere respectively after 90 minutes of occlusion and 24 hours of reperfusion. (e-h) were protein levels of IL-1β, TNF-α, IL-4 and IL-10 in ipsilateral hemisphere respectively after 90 minutes of occlusion and 24 hours of reperfusion. (i-j) the expression levels of IL-1β and TNF-α were determined using anti-IL-1β antibody or anti-TNF-α antibody (Green) immunofluorescence in ipsilateral hemisphere after 90 minutes of occlusion and 24 hours of reperfusion. Nuclear stain by Hoechst 33342 (Blue). Scale bars, 500 μm. ns, no significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Vanillin inhibited expression of pro-inflammatory cytokines by LPS stimulated primary microglia (a) The purity of primary microglia was determined using Iba-1 (Red) immunofluorescence. Nuclear stain by Hoechst 33342 (Blue). Cells were pre-treated with different treatment for 30 min before the stimulation by 100 ng/mL LPS for 24 hours individually. (b-e) were mRNA levels of IL-1 β , TNF- α , IL-4 and IL-10 in cells and (f-i) were protein levels of IL-1 β , TNF- α , IL-4 and IL-10 in cells. Scale bars, 500 μ m. Values are mean $\pm$ SD of three independent experiments. ns, no significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4

Vanillin inhibited expression of pro-inflammatory cytokines by LPS stimulated BV2 cells Cells were pre-treated with different treatment for 30 min before the stimulation by 100 ng/mL LPS for 24 hours individually. (a-d) were mRNA levels of IL-1 β , TNF- α , IL-4 and IL-10 in cells and (e-h) were protein levels of IL-1 β , TNF- α , IL-4 and IL-10 in cells. Values are mean $\pm$ SD of three independent experiments. ns, no significance. * p <0.05, ** p <0.01, *** p < 0.001.

Figure 5

Vanillin inhibited activation of microglia by inhibiting TLR4 / NF- κ B signal pathway Cells were pre-treated with different treatment for 30 min before the stimulation by 100 ng/mL LPS for 30 min individually. The protein levels of TLR4, NF- κ B p65 and phosphorylated NF- κ B p65 in primary microglia(a) and BV2 microglia (c) were measured using western blot. (b) and (d) were statistical graphs of A and C, respectively. Values are mean $\pm$ SD of three independent experiments. ns, no significance. * p <0.05, ** p <0.01, *** p < 0.001.

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