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Different effects of ferulic acid and tetramethylpyrazine on the production of superoxide anion and nitric oxide in murine macrophages

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Abstract—The effects of ferulic acid and tetramethylpyrazine, two active ingredients of the Chinese herbal medicine *Ligusticum wallichii* Franchat, on the generation of superoxide anion and nitric oxide in macrophages were studied. Ferulic acid, but not tetramethylpyrazine, scavenges superoxide anion dose-dependently. Tetramethylpyrazine inhibits the expression of the iNOS gene, and consequently decreases the formation of nitric oxide. However, ferulic acid shows no effect on iNOS expression and NO production. The results suggest that the protective effects of extracts of *Ligusticum wallichi* Franchat against ischemic injury might be due to the scavenging of superoxide anion and the regulation of NO production.

Keywords: Ferulic acid; tetramethylpyrazine; nitric oxide; superoxide; macrophage; iNOS.

INTRODUCTION

The Chinese herbal medicine *Ligusticum wallichii* Franchat (Chuan Xiong) is widely used in the treatment of ischemic stroke and arteriosclerosis by Chinese herbalists. However, by which mechanism *Ligusticum wallichii* Franchat protects the brain is still not clear. Phytochemists have investigated the components of extracts of *Ligusticum wallichii* Franchat systematically, and hundreds of components have been identified. Ferulic acid (FA) and tetramethylpyrazine (TMP) are two of the most important active ingredients; their structures are shown in Fig. 1. Among

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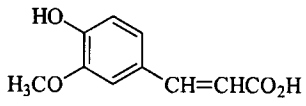
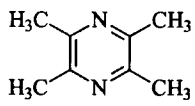


Ferulic acid



Tetramethylpyrazine

Figure 1. Molecular structure of ferulic acid and tetramethylpyrazine.

efforts to elucidate the mechanisms of their action, our previous study has shown that TMP modulates NO generation in human polymorphonuclear leukocytes [1]. FA, as well as its derivatives, shows potent antioxidant capacity in both cell-free systems and in cells [2, 3]. Considering reactive oxygen species (ROS) and NO generated by phagocytes are involved, at least in part, in the pathogenesis of ischemic stroke [4], it is possible that TMP and FA protect the brain by scavenging endogenous ROS, as well as by modulating the generation of NO. In this work, the respiratory burst of macrophages induced by N-formylmethionyl-leucyl-phenylalanine (fMLP) and the iNOS gene expression in macrophages treated with lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) were used as the experimental models for the pathophysiological generation of endogenous ROS and NO. By using electron paramagnetic resonance (EPR) spin trapping techniques, the ability of FA and TMP to scavenge $O_2^{\cdot -}$ and NO generated by macrophages was evaluated directly. The effect of TMP on the expression of iNOS gene in murine macrophages was also examined by Western blotting and RT-PCR techniques for the first time.

EXPERIMENTAL

Materials

Adult, female BALB/c mice (body weight 18–20 g; Beijing Vital River Experimental Animal Center) were used as cell sources in the experiments. RPMI 1640 medium and cell culture supplements were obtained from Hyclone. Cell-culture plastic ware was from Corning Costar (Acton, MA, USA). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), luminol, lipopolysaccharide (LPS), interferon-γ (IFN-γ) and N-formylmethionyl-leucyl-phenylalanine (fMLP) were products of Sigma. Ferulic acid (FA) was from Chendu First Pharmaceutical Factory. Tetramethylpyrazine (TMP) was from Beijing Fourth Pharmaceutical Factory. Anti-iNOS antibody was from BD Transduction Laboratories. Anti-β-actin and HRP-conjugated goat anti-mouse IgG antibodies were from Santa Cruz. The Uniq-10™ RNA purification kit was purchased from Sangon. The Access Quick™ RT-PCR kit was purchased from Promega. Other reagents were made in China.

Cell culture and drug treatment

Primary macrophages were isolated from BALB/c mice by peritoneal lavage using Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS), as described previously [5].

Cells were harvested by centrifugation at 200 × *g* for 5 min, resuspended in RPMI 1640 culture medium, plated at 1×10^7 cells per 25 cm^2 cell culture flask in 5 ml of RPMI 1640 media supplemented with 10 mM Hepes buffer, 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured in a humidified 5% CO_2 incubator at 37°C.

After incubation for 2 h, non-adherent cells were removed and remnant macrophages were cultured in RPMI 1640 containing certain concentrations of FA or TMP for 30 min. Then macrophages were subjected to different stimuli as described below.

Superoxide anion assay

The kinetic generation of $O_2^{\cdot -}$ during the respiratory burst was monitored by luminol-enhanced chemiluminescence as reported elsewhere [6]. Upon treatment with FA or TMP for 30 min, macrophages were detached from the culture flasks with rubber policemen and harvested by centrifugation. Macrophages (1×10^7 suspended in 1 ml of HBSS containing 10 μM luminol) were transferred into a quartz test tube, fitted into the detection cavity of a BPCL-IV Ultra-weak Chemiluminescence Analyzer, and equilibrated at 37°C. After the addition of 1 μM fMLP, which stimulated the respiratory burst of macrophages, the kinetic generation of luminol-dependent chemiluminescence was recorded immediately.

Superoxide anion generated by macrophages was further confirmed by EPR spin trapping technique, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin trap [7]. Briefly, 1×10^7 FA- or TMP-treated macrophages were suspended in 100 μl of HBSS containing 0.1 M of the spin trap DMPO, and then stimulated with 1 μM of fMLP, transferred into a quartz capillary and fitted into the cavity of a Bruker ER-200 D-SRC EPR spectrometer. After incubation at 37°C for 2 min, the EPR spectrum was recorded immediately with conditions described as follows: X-band; sweep width 200 G; microwave power 20 mW; 100 kHz modulation with amplitude 1 G; time constant 0.128 s.

Nitric oxide assay

iNOS gene expression and the consequent production of NO in macrophages were induced by LPS and IFN-γ. NO was detected directly by EPR spin trapping [8]. Briefly, 1×10^7 macrophages, cultured in 25-cm^2 cell-culture flasks, were incubated with certain concentrations of FA or TMP for 30 min, and treated with 1 μg/ml of LPS and 100 U/ml of IFN-γ in RPMI 1640 medium for 12 h. Then the spin trapping agent, containing 1 mM of $FeSO_4$, 5 mM of diethyldithiocarbamate sodium salt (DETC) and 5 mM of $Na_2S_2O_3$, was added into activated macrophages, which were incubated at 37°C for an additional 3 h. The paramagnetic [ON-$Fe^{2+}$$(DETC)_2$] complex was enriched by extraction with 200 μl of ethyl acetate and detected by a Bruker ER-200 D-SRC EPR spectrometer with conditions described as follows: X-band; sweep width 400 G; microwave power 20 mW; 100 kHz modulation with amplitude 3.2 G; time constant 0.128 s.

Detection of iNOS gene expression

The levels of iNOS protein were determined by Western blot [9]. In brief, 1×10^7 macrophages cultured in 25-cm^2 flasks were pretreated with FA or TMP for 30 min and then stimulated with LPS/IFN-γ for 12 h. Then cells were washed twice with PBS and lysed with 500 μl of lysis buffer (1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) at 4°C for 30 min. After centrifugation at 12 000 $\times$ g for 15 min, the supernatant was collected and protein concentration was determined by Bradford method with bovine serum albumin as standard. Each sample containing 60 μg of cellular protein was electrophoresed on 8% SDS-polyacrylamide gels and then transferred to PVDF membrane. The membrane was incubated with mouse anti-iNOS or anti-β-actin antibodies, respectively, and then incubated with HRP-conjugated goat anti-mouse IgG antibody. After four washes with 0.05% TBST, the target protein was detected by enhanced chemiluminescence.

iNOS mRNA levels in macrophages were detected by RT-PCR [10]. 1×10^7 macrophages, cultured in 25-cm^2 flasks, were pretreated with FA or TMP for 30 min and then stimulated with LPS/IFN-γ for 12 h. Total RNA was isolated with Sangon Uniq-10™ RNA purification kit according to manufacturer's instructions. RT-PCR was carried out using the Promega AccessQuick™ RT-PCR system, with primers specific for iNOS and GAPDH as the house-keeping gene. For iNOS, the primers were 5′-GTGTTCCACCAGGAGATGTTG-3′/5′-CTCCTGCCCACTGAGTTCGTC-3′, the length of the PCR product was 576 bp; for GAPDH, the primers were 5′-GAAGGGTGGGGCCAAAAG-3′/5′-GGATGCAGGGATGATGTTCT-3′, the length of the PCR product was 295 bp. PCR products were visualized by electrophoresis through a 1.5% agarose gel stained with ethidium bromide.

Statistical analysis

Each experiment was performed at least three times and the results were presented as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA). A level of $P < 0.05$ was considered significant.

RESULTS

Scavenging of superoxide anion

Upon stimulation with fMLP, respiratory burst occurs in macrophages, which is characterized by the generation of large amounts of $O_2^{\cdot -}$. The kinetic generation of $O_2^{\cdot -}$ by fMLP-stimulated macrophages could be detected using a luminol-enhanced chemiluminescence technique, as shown in Fig. 2A. Pretreatment with ferulic acid significantly inhibits the fMLP-stimulated chemiluminescence of macrophage.

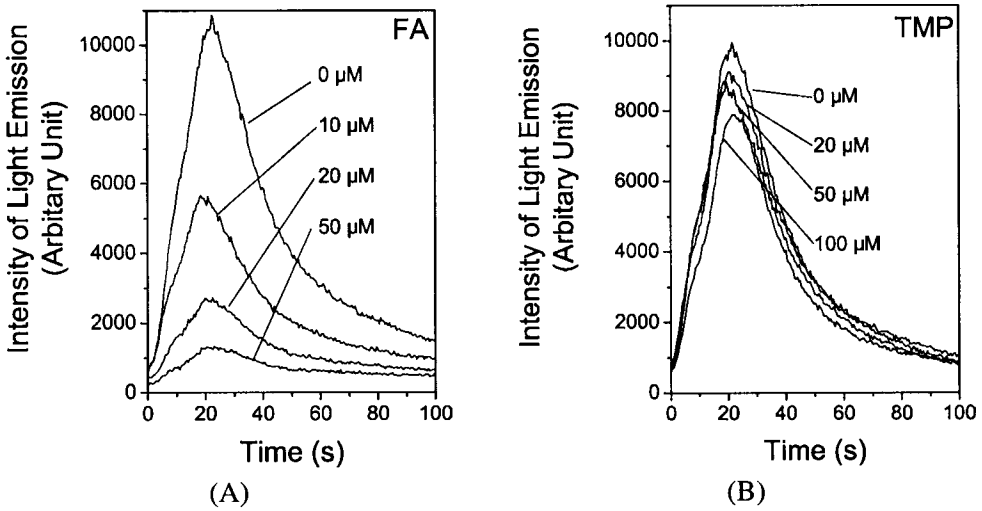


Figure 2. Kinetics of the chemiluminescence in fMLP-stimulated macrophages. 1×10^7 macrophages pretreated with FA (A) or TMP (B) for 30 min were suspended in 1 ml of HBSS containing 10 μM luminol and stimulated with 1 μM of fMLP. The kinetic generation of luminol-dependent chemiluminescence was recorded immediately.

In macrophages pretreated with 10 μM of FA, the chemiluminescence intensity decreases to 55.1 $\pm$ 8.0% of control; in macrophages pretreated with 50 μM of FA, the chemiluminescence intensity decreases to 15.0 $\pm$ 2.1% of control. Higher concentrations of TMP also decrease the intensity of chemiluminescence to some extent, as shown in Fig. 2B.

Using DMPO as the spin trap, $O_2^{\cdot -}$ generated by fMLP-stimulated macrophages could be detected directly by EPR. Figure 3A shows the typical EPR spectrum of DMPO-$O_2^{\cdot -}$ spin adduct obtained in fMLP-stimulated macrophages. FA scavenges superoxide dose-dependently as shown in Fig. 3B. In macrophages pretreated with 50 μM of FA, the signal intensity of DMPO-$O_2^{\cdot -}$ spin adduct decreases to 23.5 $\pm$ 5.3% of control. TMP shows only weak scavenging effects on superoxide at higher concentrations.

Inhibition of nitric oxide production

A three-line EPR spectrum corresponding to the [ON-Fe^{2+}(DETC)$_2$] complex at $g = 2.035$ is observed in macrophages treated with LPS/IFN-γ for 12 h (Fig. 4A), suggesting that macrophages generate nitric oxide upon stimulation. Pretreatment of macrophages with TMP suppresses the EPR signal intensity dose-dependently as shown in Fig. 4B. In macrophages pretreated with 20 μM or 50 μM of TMP, the EPR signal intensity of [ON-Fe^{2+}(DETC)$_2$] complex is 64.0 $\pm$ 5.3% and 37.7 $\pm$ 4.8% of control macrophages, respectively. However, FA shows no effect on NO production even at higher concentrations (up to 100 μM).

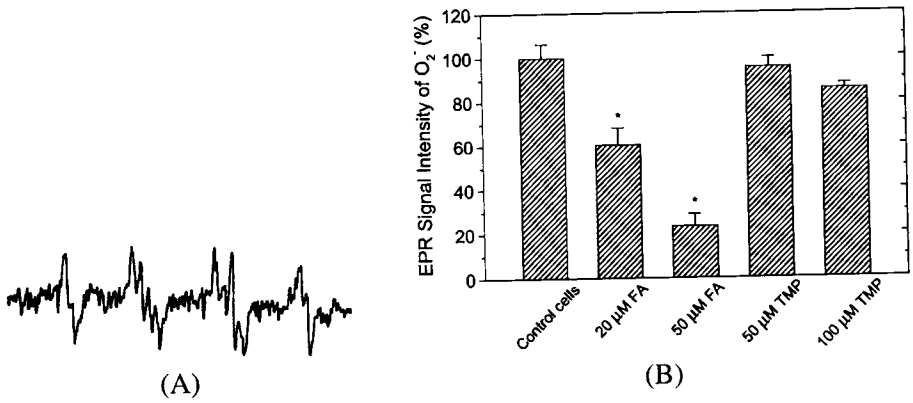


Figure 3. (A) EPR spectrum of DMPO-$O_2^{\cdot-}$ spin adducts observed in fMLP-stimulated macrophages. (B) Scavenging effects of FA and TMP on the superoxide anion. Macrophages were pretreated with different concentrations of FA or TMP for 30 min and then stimulated with 1 μM fMLP. The superoxide anion was trapped with DMPO and the EPR spectrum was recorded with Bruker ER-200 D-SRC EPR spectrometer. Data are mean $\pm$ SD, $n = 6$. $^*P < 0.05$ in comparison with control macrophages.

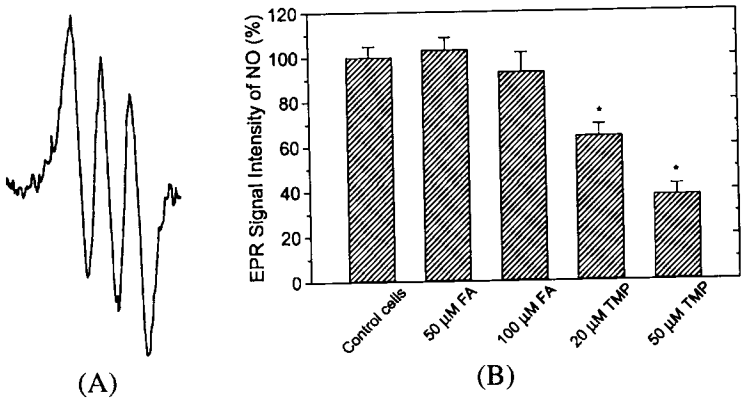
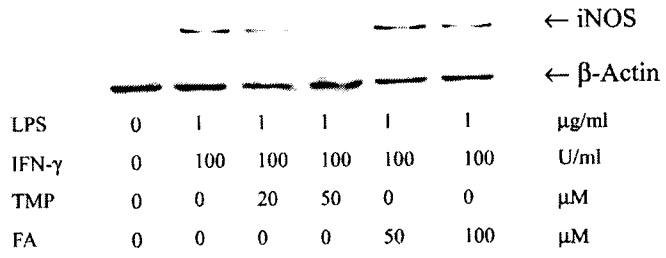


Figure 4. (A) EPR spectrum of [ON-Fe^{2+}(DETC)$_2$] complex observed in LPS/IFN-treated macrophages. (B) Inhibition effects of FA and TMP on NO production. Macrophages were pretreated with different concentrations of FA or TMP for 30 min and then treated with 1 μg/ml LPS plus 100 U/ml IFN-γ for 12 h. NO was trapped with [Fe^{2+}(DETC)$_2$] and the EPR spectrum was recorded with Bruker ER-200 D-SRC EPR spectrometer. Data are mean $\pm$ SD, $n = 6$. $^*P < 0.05$ in comparison with control macrophages.

Inhibition of iNOS gene expression

To understand the mechanisms of the inhibition of LPS/IFN-γ-induced NO production by FA or TMP, the effect of FA and TMP on the expression of the iNOS gene was studied. Immunoblotting with antibody against murine macrophage iNOS and RT-PCR for analysis of iNOS mRNA clearly shows that pretreatment of macrophages with TMP for 30 min dose-dependently inhibits the LPS/IFN-γ-



LPS	0	1	1	1	1	1	μg/ml
IFN-γ	0	100	100	100	100	100	U/ml
TMP	0	0	20	50	0	0	μM
FA	0	0	0	0	50	100	μM

Figure 5. Effects of FA and TMP on iNOS protein in macrophages. Macrophages were pretreated with FA or TMP for 30 min and then stimulated with 1 μg/ml LPS plus 100 U/ml IFN-γ for 12 h. The expression of iNOS protein was determined by western blot.

LPS	0	1	1	1	1	1	μg/ml
IFN-γ	0	100	100	100	100	100	U/ml
FA	0	0	50	100	0	0	μM
TMP	0	0	0	0	20	50	μM

Figure 6. Effects of FA and TMP on iNOS mRNA in macrophages. Macrophages were pretreated with FA or TMP for 30 min and then stimulated with 1 μg/ml LPS plus 100 U/ml IFN-γ for 12 h. The expression of iNOS mRNA was determined by RT-PCR.

mediated induction of iNOS protein and mRNA, as shown in Figs 5 and 6, respectively, whilst FA shows no apparent effects on the expression of the iNOS gene.

DISCUSSION

Macrophages are one of the key mediators of the immune system, which generate large quantities of superoxide in response to a variety of stimuli, by a coordinated sequence of biochemical reactions known as the respiratory burst [11]. At the same time, upon stimulation by certain cytokines such as IFN-γ, interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α), the iNOS gene of macrophages is induced, by which large amount of NO is generated [12]. The regulation of iNOS expression is complex, but appears to occur primarily at the level of transcription. The murine iNOS promoter contains regulatory regions incorporating binding sites for nuclear factor-κB (NF-κB), activator protein 1 (AP-1), CCAAT/enhancer binding protein (C/EBP), TNF response element and IFN-related transcriptional factors [13, 14]. Simultaneously generated superoxide and NO may cause the formation of peroxynitrite and other potentially oxidants, which are required for the microbicidal properties of macrophages. However, excessive production of superoxide, NO and peroxynitrite by macrophages has been implicated in a number of pathological processes, such as the development of cardiovascular diseases,

neurodegenerative disorders and cancer [4]. Accordingly, drugs that modulate ROS or NO generation may have beneficial effects in the treatment of related diseases.

In the present investigation, fMLP-stimulated respiratory burst of macrophage was used as the experimental model for $O_2^{\cdot -}$ generation. NO production by macrophages was induced by LPS/IFN-γ exposure. Using this model, the effects of FA and TMP, two of the most important active ingredients of the Chinese traditional herb *Ligusticum wallichii* Franchat, on $O_2^{\cdot -}$ and NO generation, were studied. The results reveal that FA could directly scavenge the endogenous $O_2^{\cdot -}$ effectively. TMP also shows scavenging effect against $O_2^{\cdot -}$ at higher concentrations. Interestingly, TMP, but not FA, decreases the generation of NO dose-dependently. To understand the molecular mechanism of the modulation of NO production by TMP, the effects of TMP on iNOS gene expression were studied. TMP dose-dependently decreases the iNOS mRNA and protein in LPS/IFN-γ-treated macrophages, suggesting that TMP decreases the NO production by down-regulation the expression of iNOS gene. This result is in accordance with our previous data that TMP modulate the generation of NO in human polymorphonuclear leukocytes [1].

Our previous study shows that FA is a potent ROS scavenger in both cell-free systems and in cultured neurons, and is almost as effective as α-tocopherol [2, 3]. In the present investigation, the direct scavenging of $O_2^{\cdot -}$ in fMLP-stimulated macrophages by FA is reported. We report the inhibition of iNOS gene expression and the consequent decrease of NO production by TMP in primary cultures of macrophages for the first time. Considering that both FA and TMP are important ingredients of the Chinese herb *Ligusticum wallichii* Franchat, the above data suggest that the extract of the Chinese herb *Ligusticum wallichii* Franchat is an effective regulator on NOS activity, as well as a potent ROS scavenger. The effective protection of Chinese herb *Ligusticum wallichii* Franchat against ischemic brain injury and arteriosclerosis might be due to its scavenging of ROS and regulation on NO production, and consequent prevention of peroxynitrite formation.

Acknowledgements

The first two authors contributed equally to this work.

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