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Tetramethylpyrazine scavenges superoxide anion and decreases nitric oxide production in human polymorphonuclear leukocytes

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Abstract

Tetramethylpyrazine is one of the active ingredients of the Chinese herb *Ligusticum wallichii* Franchat. By electron spin resonance spin trapping methods, effects of tetramethylpyrazine on superoxide anion and nitric oxide generated by human polymorphonuclear leukocytes were studied. During the respiratory burst of polymorphonuclear leukocytes induced by N-formylmethionyl-leucyl-phenylalanine, tetramethylpyrazine scavenges superoxide anion dose-dependently, and decreases the production of nitric oxide significantly, but shows no influence on oxygen consumption. These results suggest that the effective protection of tetramethylpyrazine against ischemic brain injury might be due to its scavenging of reactive oxygen species and regulation on nitric oxide production, and consequent prevention of peroxynitrite formation.
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Introduction

The important role of phagocytic cells, including polymorphonuclear leukocytes (PMN), is to protect organisms against invading pathogens by various functions such as phagocytosis, respiratory burst, and production of nitric oxide (NO). However, the endogenous superoxide anion ($O_2^{\cdot-}$) and NO generated by

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activated phagocytic cells can also act as double-edged swords and may cause oxidative damage to organisms. Recently emerging evidences implicate a role for $O_2^{\cdot-}$ and NO generated from PMN in the pathogenesis of cerebral ischemia [1,5]. Under cerebral ischemia conditions, the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex of PMN is activated by certain cytokines and thus releases large amounts of $O_2^{\cdot-}$ into both the phagocytic vacuoles and the extracellular medium [9]. On the other hand, the nitric oxide synthase (NOS) of PMN is also activated during cerebral ischemia, high concentration of NO is released and then reacts rapidly with $O_2^{\cdot-}$ to form peroxynitrite (ONOO$^-$) [18]. Peroxynitrite is a potent oxidant that can oxidize almost all cellular components and thus induces apoptosis in neighboring neuronal cells [12]. Accordingly, drugs that can scavenge $O_2^{\cdot-}$ and/or NO, and prevent the formation of peroxynitrite in PMN might be potential neuroprotectors against cerebral ischemia.

Tetramethylpyrazine (TMP), which is widely used in the treatment of ischemic stroke by Chinese herbalists, is one of the most important active ingredients of the traditional Chinese herbal medicine *Ligusticum wallichii* Franchat (Chuan Xiong). TMP can permeate the blood–brain barrier and can be enriched in the brain, especially in the brainstem. The clinical dosage of TMP for the treatment of stroke is varied from 160 to 320 mg via intravenous injection. However, by which mechanisms does TMP protect the brain is still not clear. Among efforts to elucidate the mechanisms of TMP's action, some studies have shown that TMP inhibits platelet aggregation via nitric oxide-mediated pathways [11]. TMP also shows strong anti-thrombotic activity [8]. Huang and co-workers reported that TMP inhibits the chemiluminescence of PMN induced by phorbol myristate acetate, and scavenges $O_2^{\cdot-}$ generated by xanthine–xanthine oxidase system as well as hydroxyl radicals produced by ascorbate–copper ion–zymosan system [3]. Considering reactive oxygen species (ROS) and NO generated by PMN are involved, at least in part, in the pathogenesis of ischemic stroke, it is possible that TMP protect the brain by scavenging endogenous ROS as well as modulating the generation of NO. However, little is known about the direct effects of TMP on endogenous ROS and NO. In this work, the N-formylmethionyl-leucyl-phenylalanine (fMLP)-induced respiratory burst of PMN was used as the experimental model for the pathophysiological generation of endogenous ROS and NO. By using electron spin resonance (ESR) spin trapping technique as well as ESR oxymetry, the ability of TMP to scavenge $O_2^{\cdot-}$ generated by PMN was evaluated directly. The effects of TMP on NO production in activated PMN were also examined for the first time.

Materials and methods

Reagents

Tetramethylpyrazine was a generous gift from Beijing Fourth Pharmaceutical Factory. Percoll was purchased from Amersham Pharmacia. N-formylmethionyl-leucyl-phenylalanine, 5,5-dimethyl-1-pyrro-line-N-oxide, 3-carbamoyl-2,2,5,5-trtramethyl-3-pyrroline-1-yloxy and other reagents were from Sigma.

Isolation of PMN

Whole blood samples of healthy donors were obtained from the Red Cross of Beijing. PMN were isolated by a one-step discontinuous Percoll gradient centrifugation and purified by hypotonic lysis at

4 °C as described [18]. The purified PMN (> 95% viability determined by Trypan blue exclusion) were resuspended in Hanks' balanced salt solution (HBSS).

Detection of superoxide anion

Superoxide anion production from PMN was detected directly by ESR spin trapping technique [16]. Briefly, 10^7 PMN suspended in HBSS were preincubated with or without TMP at 37 °C for 5 min, stimulated with 100 nM of fMLP at 37 °C for 15 min, mixed with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.1 M), transferred into quartz capillary, and fitted into the cavity of ESR spectrometer. After incubation at 37 °C for 2 min, the ESR spectrum was recorded immediately.

ESR oxymetry

Effect of TMP on oxygen consumption rate of PMN was measured by ESR oxymetry [17]. In brief, 2×10^6 PMN stimulated with 100 nM of fMLP at 37 °C for 15 min were mixed with 0.1 mM of 3-carbamoyl-2,2,5,5-trtramethyl-3-pyrroline-1-yloxy (CTPO), and then sealed into quartz capillary for ESR measurement. The ESR spectra were recorded 2 min, 8 min, and 14 min after the addition of CTPO. The K value calculated from the ESR spectra parameters was used as the index of oxygen consumption rate. K value was calculated by the following equation:

$$K = b + c/2a$$

Detection of NO

NO produced from PMN was detected directly by ESR spin trapping [19]. Briefly, 2×10^7 PMN suspended in HBSS containing 100 μM of L-arginine were preincubated with or without TMP at 37 °C
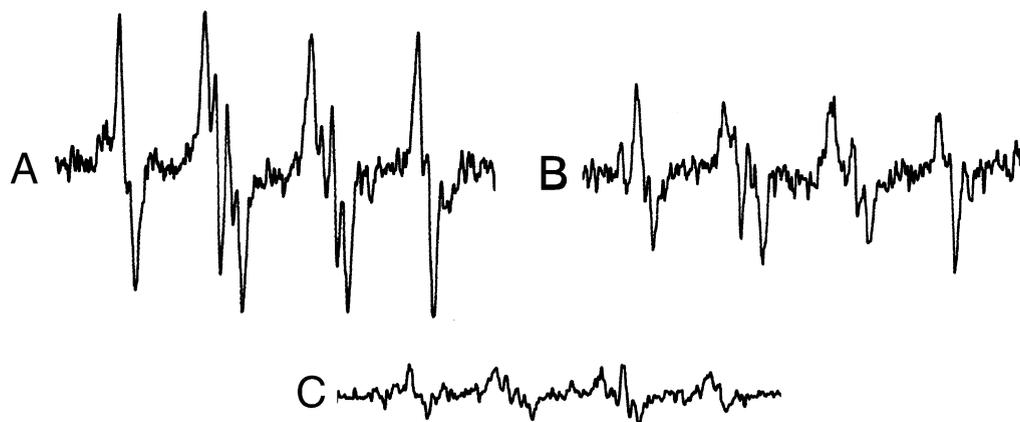

Fig. 1. Direct ESR measurement of superoxide anion generated by fMLP-stimulated human PMN. PMN were preincubated with or without TMP at 37 °C for 5 min and then stimulated with 100 nM of fMLP for 15 min. Then the superoxide anion generated by PMN was determined by ESR spin technique with DMPO (0.1 M) as the spin trap. A, without TMP pretreatment; B, preincubated with 0.09 mM of TMP; C, preincubated with 0.18 mM of TMP.

Table 1
Effects of TMP on superoxide anion generation in fMLP-stimulated human PMN

Group	fMLP only	with 0.09 mM TMP	with 0.18 mM TMP
Relative amount of $O_2^{\cdot-}$	100 ± 4.2	$45.7 \pm 6.3*$	$0*$

Data are presented as mean $\pm$ SD, n = 4.
*$p < 0.05$ in comparison with cells treated with 100 nM of fMLP only.

for 5 min, and stimulated with 100 nM of fMLP [6] at 37 °C for 15 min. Then the spin trapping agent containing 1 mM of $FeSO_4$, 5 mM of diethyldithiocarbamate sodium salt (DETC) and 5 mM of $Na_2S_2O_3$ were added into the cells and the cells were incubated at 37 °C for 1 h. The paramagnetic ON-Fe(DETC)$_2$ complex was enriched by extraction with 200 µl of ethyl acetate and determined by ESR.

ESR measurement conditions

All ESR spectra were recorded by a computerized Bruker ER200D-SRC spectrometer. The measurement conditions were listed as followed: X-band; sweep width 200 G (for detection of $O_2^{\cdot-}$), 400 G (for
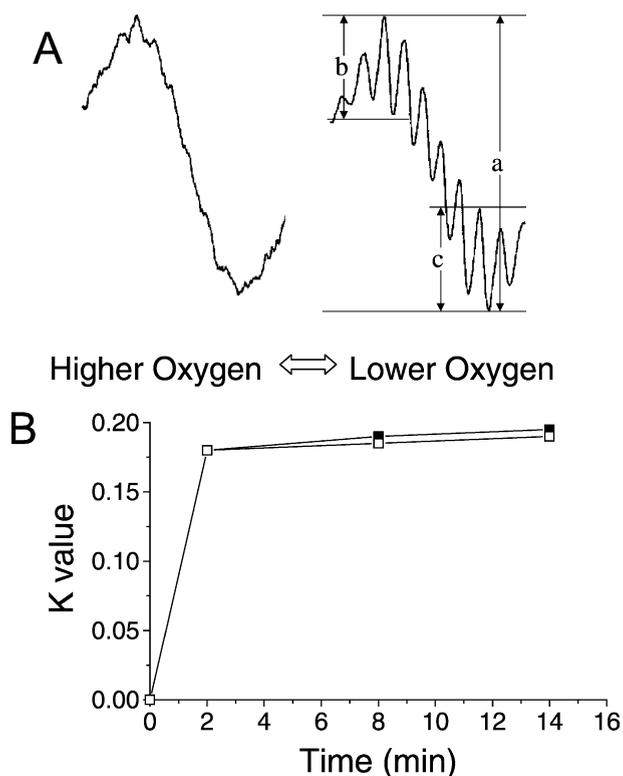

Fig. 2. A. Typical ESR spectra of 0.1 mM of CTPO spin probe in PMN suspension containing higher or lower concentrations of molecular oxygen. B. Effects of TMP on oxygen consumption rate during the respiratory burst of human PMN stimulated with 100 nM of fMLP. K values were calculated from the ESR spectrum by the equation K = b + c / 2a and were used as the index of oxygen consumption rate. Solid square: PMN without TMP pretreatment; open square: PMN pretreated with 0.18 mM of TMP.

detection of NO) or 20 G (ESR oxymetry); microwave power 20 mW (for detection of $O_2^{\cdot-}$ and NO) or 1 mW (ESR oxymetry); 100 kHz modulation with amplitude 1 G (for detection of $O_2^{\cdot-}$), 3.2 G (for detection of NO) or 0.05 G (ESR oxymetry); time constant 0.128 s.

Results

TMP scavenges $O_2^{\cdot-}$

Upon stimulation with fMLP, respiratory burst occurs in PMN, which is characterized by the generation of large amount of $O_2^{\cdot-}$ and the respiratory burst-associated oxygen consumption as well. Using DMPO as the spin trap, $O_2^{\cdot-}$ generated by fMLP-stimulated PMN could be detected directly as shown in Fig. 1A. TMP inhibits the DMPO-$O_2^{\cdot-}$ spin adduct dose-dependently as shown in Fig. 1B and 1C as well as in Table 1. This might be due to two different mechanisms: first, TMP might scavenge $O_2^{\cdot-}$ directly; second, TMP might decrease the generation of $O_2^{\cdot-}$ indirectly by inhibiting the respiratory burst of PMN. In order to solve this question, the ESR oxymetry was used to explore the effect of TMP on the respiratory burst of stimulated PMN. Fig. 2A shows the typical ESR spectra of the ESR oxymetry spin probe CTPO. The K values calculated from the ESR spectra can be used as indexes for oxygen consumption rates. According to results shown in Fig. 2B, there are no differences between the oxygen consumption rates of PMN with or without TMP pretreatment. These results indicate that TMP does not affect the oxygen consumption during the respiratory burst. In other words, TMP does not influence the
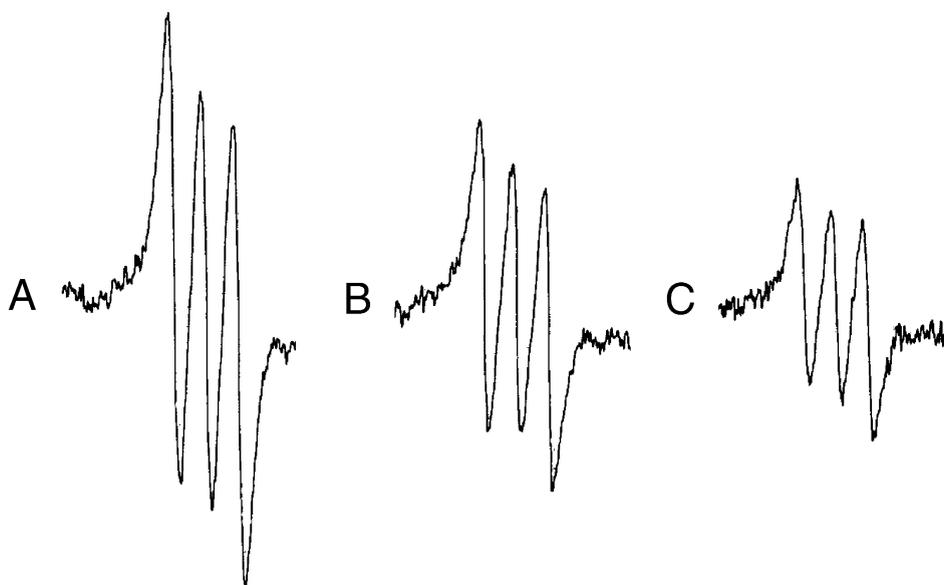

Fig. 3. Direct ESR measurement of NO generated by fMLP-stimulated human PMN. PMN were preincubated with or without TMP at 37 °C for 5 min and then stimulated with 100 nM of fMLP for 15 min. Then the NO generation in PMN was determined by ESR spin technique with Fe(DETC)$_2$ (1 mM) as the spin trap. A, without TMP pretreatment; B, preincubated with 0.09 mM of TMP; C, preincubated with 0.18 mM of TMP.

Table 2
Effects of TMP on NO generation in fMLP-stimulated human PMN

Group	fMLP only	with 0.09 mM TMP	with 0.18 mM TMP
Relative amount of NO	100 ± 8.2	65.3 ± 4.3*	35.7 ± 3.5*

Data are presented as mean $\pm$ SD, n = 4.
  *$p < 0.05$ in comparison with cells treated with 100 nM of fMLP only.

production of $O_2^{\cdot-}$ in fMLP-stimulated PMN. TMP decreases the formation of DMPO-$O_2^{\cdot-}$ spin adducts by scavenging the superoxide anion directly.

TMP inhibits the formation of NO

Stimulation by fMLP also triggered the formation of NO in PMN, as measured by ESR spin trapping technique (Fig. 3A). TMP suppresses the signal intensity of ON-Fe(DETC)$_2$ complex dose-dependently as shown in Fig. 3B and 3C as well as in Table 2. This also might be due to two different mechanisms: first, TMP might scavenge NO directly; second, TMP might decrease the generation of NO by inhibiting the activity of NOS. Preliminary experiments show that TMP has no direct scavenging effects on NO as measured by both ESR spin trapping and oxyhemoglobin assay [2] (data not shown). So it can be concluded that TMP decreases the signal intensity of ON-Fe(DETC)$_2$ complex by inhibiting the production of NO.

Discussion

NO shows both protective and toxic effects during cerebral ischemia. In the early stages of cerebral ischemia, the beneficial vascular effects of endothelial NOS (eNOS) outweigh the neurotoxic potential of neuronal NOS (nNOS). In the late stages of cerebral ischemia (> 6 h), inducible NOS (iNOS) is expressed in the setting of post-ischemic inflammation, which would lead to neurotoxic effect [4]. Many kinds of blood cells such as eosinophils, platelets, neutrophils, monocytes and macrophages can generate NO. Among them, PMN constitute an important proportion and are also the major participants in a number of pathological conditions with suggestive involvement of NO. It had been reported that PMN can synthesize NO at rates similar to endothelial cells for a long period [10]. Also, PMN can produce ROS such as superoxide anion via NADPH oxidase during the respiratory burst. Simultaneous generation of $O_2^{\cdot-}$ and NO may result in the formation of peroxynitrite, a potent oxidant which is destructive to ischemic brain [5]. Considering that most patients with ischemic stroke arrive at the emergency room several hours after the onset of symptoms, ROS generated by NAPPH oxidase and NO generated by iNOS are involved in neuronal injury. Accordingly, drugs that can regulate the generation of ROS and NO would be extremely valuable. In the present investigation, fMLP-stimulated PMN was used as the experimental model for PMN activation during cerebral ischemia. Upon stimulation with fMLP, PMN produces large amounts of $O_2^{\cdot-}$ and NO, which is in accordance with the previous observations in macrophages [7,18]. Using this model, the effects of TMP, one of the active ingredients of the Chinese traditional herb *Ligusticum wallichii* Franchat, on $O_2^{\cdot-}$ and NO generation were studied. The results of present investigation reveal that TMP could directly scavenge the endogenous $O_2^{\cdot-}$ effectively, and decrease the generation of NO dose-dependently. In order to work

out by which kinds of NOS do PMN generate NO, the influence of calcium ion on NO generation in PMN is studied. Depletion of extracellular calcium ion does not influence the generation of NO; increasing the intracellular calcium ion level by the calcium ionphore A23187 also does not influence the generation of NO (data not shown). These suggested that NO is synthesized via calcium-independent iNOS in activated PMN.

It had been reported that TMP could inhibit the expression of iNOS in the lung and aorta, and mitigate the delayed circulatory failure caused by endotoxic shock in rats [13]. However TMP also stimulates the expression of eNOS in human platelets [11]. TMP shows potent ROS-scavenging properties in both cell-free systems and cultured neurons and is almost as effective as α-tocopherol [14,15]. In the present investigation, the direct scavenging of $O_2^{\cdot-}$ and decrease of NO generation in fMLP-stimulated PMN by TMP is studied by ESR spin trapping for the first time. Experimental data suggest that TMP is an effective regulator on NOS activity as well as a potent ROS scavenger. The effective protection of TMP against ischemic brain injury might be due to its scavenging of ROS and regulation on NO production, and consequent prevention of peroxynitrite formation.

Acknowledgements

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