On the Role of Hydroxyl Radical and the Effect of Tetrandrine on Nuclear Factor-κB Activation by Phorbol 12-Myristate 13-Acetate

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Abstract. Nuclear factor κB (NF-κB) is considered to be an important target for therapeutic intervention because of its role in the regulation of proinflammatory and profibrotic mediators. The present study examined the role of hydroxyl (•OH) radical and the effect of tetrandrine, an alkaloid extracted from the Chinese medicinal herb *Stephania tetrandra*, on NF-κB activation by a tumor promoter, phorbol 12-myristate 13-acetate (PMA) in human lymphoid T cells (ie, Jurkat cells). Exogenous superoxide dismutase (SOD) enhanced the NF-κB activation by PMA, while catalase blocked it. Formate, a scavenger of •OH radical, also was inhibitory, as was deferoxamine, a metal chelator. These data suggest an important role of •OH radical in PMA-induced NF-κB activation. Incubation of the cells with tetrandrine prior to the stimulation of the cells was found to inhibit PMA-induced NF-κB activation. Tetrandrine activity was so potent that 50 μM of tetrandrine was sufficient to inhibit activation of NF-κB completely. Electron spin resonance (ESR) spin trapping was used to investigate the antioxidant action of tetrandrine using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap. Tetrandrine is an antioxidant for both •OH and superoxide (O_2^-)radicals. The reaction rate constant of tetrandrine with •OH is 1.4×10^{10} M^{-1} sec^{-1}, which is comparable with several well established antioxidants, such as ascorbate, glutathione, and cysteine. The Fenton reaction ($Fe(II) + H_2O_2 \longrightarrow Fe(III) + \bullet OH + OH^-$) and xanthine/xanthine oxidase were used as sources of •OH and O_2^- radicals. The free radical scavenging activity of tetrandrine is responsible for its inhibition of PMA-induced NF-κB activation.

Keywords: *Hydroxyl radical, tetandrine, NF-κB, free radical reactions, phorbol ester, superoxide radical*

Introduction

Tetrandrine is a bis-benzyl-isoquinoline alkaloid which can be extracted from the Chinese medicinal herb *Stephania tetrandra*. Tetrandrine exhibits various pharmacological actions [1-3], including inhibition of Na^+-K^+-ATPases [1] as well as anti-inflammatory [2,3] and immunosuppressive [4] activities. These actions inhibited stimulant-induced production and release of inflammatory mediators such as platelet-activating factor, interleukin-1 [3], and tumor necrosis factor [5]. As with other natural products, the mechanisms of tetrandrine's pharmacological actions are still largely unknown. Recent biomedical advances have enabled investigators to study the molecular mechanisms underlying the therapeutic actions associated with this medicine. It has been demonstrated that the anti-inflammatory effect of tetrandrine is most likely due to its inhibitory effects on immune cells such as B cells, mast cells, monocytes, neutrophils, and alveolar macrophages [5-7]. It appears that various actions of tetrandrine are paralleled by its multiple regulatory effects at the molecular level [8,9].

In the present study, we investigate the effect of tetrandrine on NF-κB activation induced by phorbol 12-myristate 13-acetate (PMA). NF-κB is an ubiquitous transcription factor whose active form is composed of two different subunits, p50 and p65 [10,11]. Although NF-κB is found in many different cell types and tissues, it has been characterized best in

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cells of the immune system, such as pre-B cells, mature B and T lymphocytes, macrophages, and monocytes [11]. The properties of NF-κB include its rapid post-translation activation in response to many pathogenic signals, its direct participation in cytoplasmic/nuclear signaling, and its potency to activate transcription of a great variety of genes encoding immunologically relevant proteins [11]. Because of its role in the regulation of a variety of proinflammatory and profibrotic mediators of host defense, NF-κB is considered to be an important target for therapeutic intervention [11].

While the exact mechanism of NF-κB activation remains to be elucidated, recent studies have reported that DNA binding and transactivation activities of NF-κB were strongly induced by H_2O_2 [12,13]. H_2O_2 also potentiated the activation of NF-κB by phorbol ester [14,15]. O_2^- was considered to be indirectly involved as it can be a source of H_2O_2 [14]. It is known that H_2O_2 can be converted to the very reactive •OH radical species, especially in the presence of metal ions [16]. Although there have been intensive studies on the mechanism of NF-κB activation by various reagents, especially PMA and H_2O_2, the role of •OH remains to be elucidated.

In this report, we show that the •OH radical plays an important role in PMA-induced NF-κB activation. Tetrandrine is a potent inhibitor of NF-κB activation induced by PMA. Tetrandrine is an antioxidant, and this property of tetrandrine may account for its inhibitory effect.

Materials and Methods

Materials. Iron(II) chloride ($FeCl_2$), hydrogen peroxide (H_2O_2), sodium formate, deferoxamine, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Aldrich (Milwaukee, WI). Superoxide dismutase (SOD), catalase, NADPH, xanthine, xanthine oxidase, diethylenetriaminepentaacetic acid (DETAPAC), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Tetrandrine (>98% purity) was a gift from Dr. Changgeng Mo (Institute of Occupational Medicine, Beijing, China). Its chemical structure is shown in Fig. 1. Tetrandrine was first dissolved in HCl solution (pH 1.5) and then the solution was adjusted to pH 3 by adding NaOH. The tetrandrine solution was then added to the sample containing phosphate-buffered solution (final solution, pH 7.4). Chelex-100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). Phosphate buffered solution (10 mM, pH 7.4) was treated with Chelex-100 to remove putative metal ion contaminants. DMPO solutions were purified using activated charcoal until free radical impurities disappeared as verified by ESR spectroscopy.

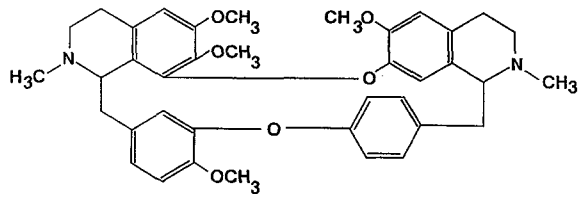

Fig. 1. The chemical structure of tetrandrine.

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA Synthesizer (Applied Biosystems, Model 392, Foster City, CA). A NF-κB binding sequence,

(5'- GAAATTCCAAAGAGTCATCAGA-3'),

from the promoter region of the human IL-2 receptor α chain gene was used to synthesize a NF-κB binding oligonucleotide. The synthesized single-stranded oligonucleotide was deprotected overnight at 50°C, dried in a speed vacuum, and then resolved in the TE buffer. Complimentary strands were denatured at 80°C for 5 min and annealed at room temperature. The double-stranded probe was labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL) using Klenow fragment (Bethesda Research Laboratories, Gaithersburg, MD).

Cell lines. Jurkat cells ($CD4^+$ human lymphoblast cell line) were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 U/ml penicillin-streptomycin (complete medium). Cells ($2x10^6$/ml) were used for treatments in this study.

Nuclear extraction. Nuclear extracts were prepared according to the method described earlier [17,18]. Briefly, 2×10^7 cells were treated with 500 µl lysis buffer on ice for 4 min. Composition of the lysis buffer is 50

mM KCl, 0.5% Nonidet P40 (NP-40), 25 mM N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES), pH 7.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 20 μg/ml aprotinin, and100 μM dethiothreitol (DTT). After 1 min centrifugation at 14,000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclei were washed once with the same buffer without NP-40, resuspended in 300 μl volume of extraction buffer (500 mM KCl, 10% glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin and DTT as the lysis buffer), and pipetted several times. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at -70°C. The protein concentration was determined by a bisinchoninic acid assay kit (Pierce, Rockford, IL).

Electrophoretic mobility shift assay (EMSA). The DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 1 μg Poly dI.dC (Sigma, St. Louis), 3 μg nuclear protein extract, 3 μg BSA, 4 x 10^4 cpm of ^{32}P-labeled oligonucleotide probe, and 12 μl of reaction buffer. This mixture was incubated on ice for 10 min in the absence of the radiolabelled probe, then incubated for 20 min at room temperature in the presence of radiolabelled probe. After the incubation, the DNA-protein complexes were resolved on a 5% acrylamide gel (National Diagnostics, Atlanta, GA) that had been pre-run at 110 V for 1 hr with 0.5x tris boric acid EDTA (TBE) buffer. The loaded gel was run at 210 V for 90 min, then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) for autoradiography. The film was developed after overnight exposure at -70°C.

ESR measurements. ESR spin trapping was used for detecting short-lived free radicals. This technique involves the addition-type reaction of a shorted-lived free radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radical trapped, and hyperfine splittings of the spin adduct are generally characteristic of the original short-lived, trapped radical. All ESR measurements were conducted using a Varian E4 ESR spectrometer and a flat cell assembly.

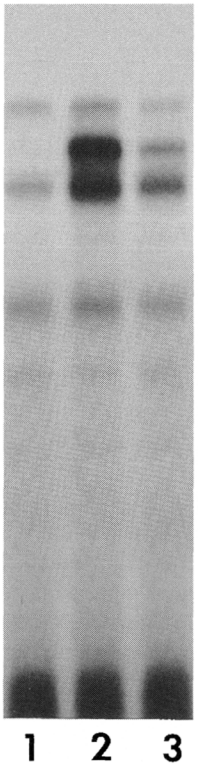

Fig. 2. Inhibition of PMA-induced NF-κB activation by tetrandrine. Jurkat cells were adjusted to a density of 2 x 10^6/ml and incubated for 3 hr with different treatments, then subjected to extraction of the nuclear proteins as stated in the Methods. PMA (10 ng/ml) was applied to the cells, and DNA binding activity of the NF-κB protein was detected with a probe of ^{32}P labeled double-stranded NF-κB binding oligonucleotide in an EMSA assay. Lane 1, untreated cells; lane 2, cells + 10 ng/ml PMA; lane 3, cells + 10 ng/ml PMA + 50 μM tetrandrine.

Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. The relative radical yield (concentration) was estimated by multiplying half of the peak-to-peak height by $(\Delta H_{pp})^2$ (where ΔH_{pp} represents peak-to-peak width). Reactants were mixed in test tubes in a total final volume of 450 μl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. All experiments were performed in ambient air and at room temperature, except as indicated.

Results

NF-κB activation. Jurkat cells were used to detect PMA-induced NF-κB activation. The cells were incubated in the presence of various reagents for 3 hr and then harvested for extraction of the nuclear proteins. These nuclear proteins were analyzed by the electrophoretic mobility shift assay (EMSA) for the DNA binding activity of NF-κB. As shown in Fig. 2,

lane 2, the NF-κB binding activity was dramatically induced in Jurkat cells by PMA. Tetrandrine depressed the PMA-induced NF-κB activation (Fig. 2, lane 3). The untreated Jurkat cells exhibited minimal NF-κB activation (Fig. 2, lane 1).

To investigate the role of •OH in PMA-induced NF-κB activation, the effects of exogenous SOD and catalase were investigated. Fig. 3, lanes 1 and 2, shows untreated and PMA treated cells. SOD enhanced the PMA-induced NF-κB activation (Fig. 3, lane 3) while catalase inhibited it (Fig. 3, lane 4). Deferoxamine, a metal chelator, exhibited minimal inhibition (Fig. 3, lane 5). Formate, a scavenger of •OH radicals, exhibited strong inhibition (Fig. 3, lane 6).

Scavenging of •OH and O_2^- by tetrandrine. The Fenton reaction (Fe(II) + H_2O_2 → Fe(III) + •OH + OH^-) was used as a source of •OH radicals to investigate the •OH scavenging activity of tetrandrine. Because of the short lifetime of •OH radical, a spin trapping technique was utilized to measure its generation using DMPO as the spin trap. As shown in Fig. 4a, an aqueous solution containing Fe(II), H_2O_2, and DMPO, in a phosphate buffered solution (pH 7.4), generated a 1:2:2:1 quartet with hyperfine splittings of $a_N = a_H = 14.9$ G. Based on these splitting

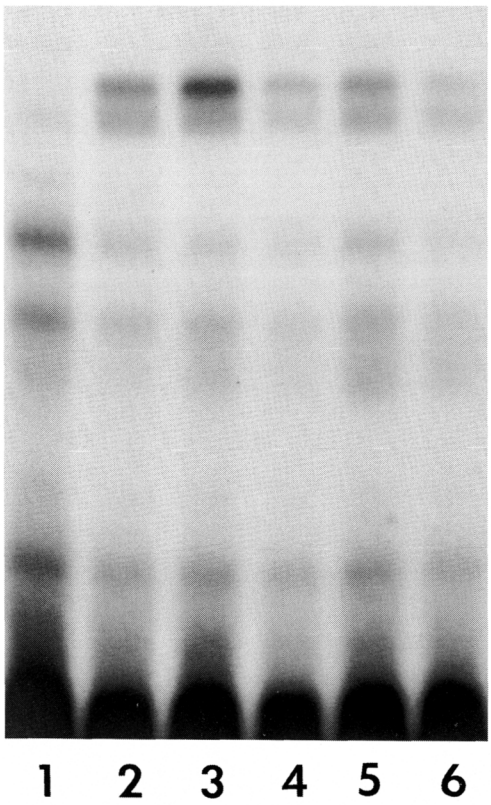

Fig. 3. Effects of exogenous antioxidants and metal chelators on PMA-induced NF-κB activation. Lane 1, untreated cells; lane 2, cells + 10 ng/ml PMA; lane 3, cells + 10 ng/ml PMA + 500 units/ml SOD; lane 4, cells + 10 ng/ml PMA + 10,000 units/ml catalase; lane 5, cells + 10 ng/ml PMA + 8 μM sodium formate; lane 6, cells + 10 ng/ml PMA + deferoxamine. Other experimental conditions were the same as those in Fig. 2.

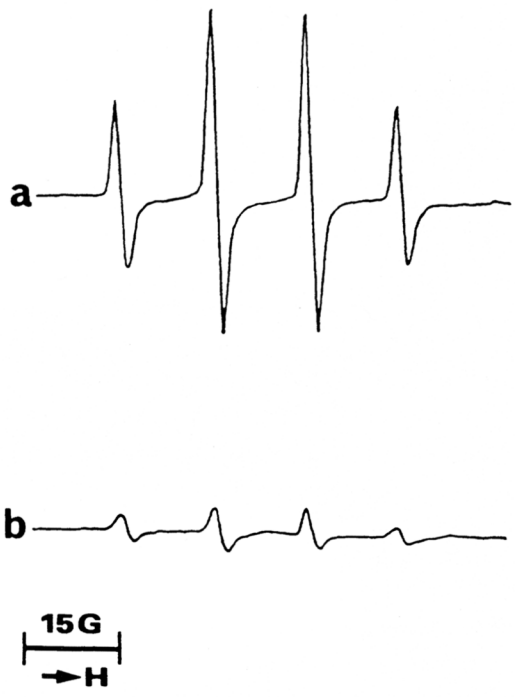

Fig. 4. (a) ESR spectrum recorded 2 min after mixing 2.5 mM $FeCl_2$, 10 mM H_2O_2, and 75 mM DMPO in a pH 7.4 phosphate-buffered solution. (b) Same as (a) but with 100 mM tetrandrine added. The spectrometer settings were: receiver gain, 1.25×10^5; modulation amplitude, 1.25 G; magnetic field, 3500 ± 100 G; scan time, 2 min.

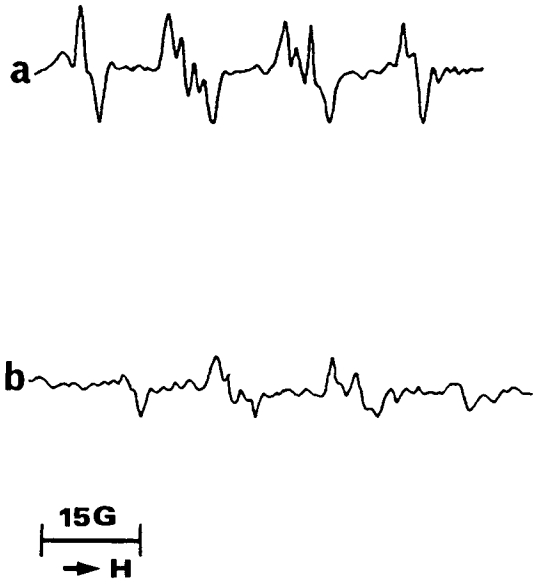

Fig. 5. (a) ESR spectrum recorded after mixing 0.05 units/ml xanthine oxidase, 0.3 mM xanthine, 0.3 mM DETAPAC, and 60 mM DMPO in a pH 7.4 phosphate-buffered solution. (b) Same as (a) but with 60 mM tetrandrine added. The spectrometer settings were: receiver gain, 4 x 10^5; modulation amplitude, 0.8 G; magnetic field, 3500 ± 100 G; scan time, 4 min; time constant.

constants, the 1:2:2:1 quartet was assigned to a DMPO/•OH adduct as evidence for •OH generation. As may be noted from Fig. 4b, tetrandrine effectively reduced the intensity of the DMPO/•OH spin adduct signal. The control, which has an equivalent volume of tetrandrine vehicle (without tetrandrine), did not alter the spectral intensity (data not shown), demonstrating that the apparent effects of tetrandrine were not due to its vehicle.

The spin trapping competition experiments were performed to calculate the reaction rate constant of tetrandrine and •OH radical as reported earlier [19,20]. The rate constant was found to be 1.4 x $10^{10}M^{-1}sec^{-1}$, which is comparable with several well established antioxidants [21], such as ascorbate (1.2 x $10^{10}M^{-1}sec^{-1}$), reduced glutathione (1.5 x $10^{10}M^{-1}sec^{-1}$), and cysteine (1.3 x $10^{10}M^{-1}sec^{-1}$).

Xanthine and xanthine oxidase were used as a source of O_2^-. As shown in Fig. 5a, a mixture of xanthine and xanthine oxidase in the presence of DMPO generated a spin adduct spectrum. Analysis of this spectrum shows hyperfine splittings of a_N = 14.2 G, a_H = 11.5 G and a_{H^-} = 1.2 G. These splittings are typical of the DMPO/O_2^- adduct. Tetrandrine reduced the intensity of DMPO/O_2^- (Fig. 5b).

Discussion

In this study, we have demonstrated that •OH radicals play an important role in PMA-induced DNA binding activity of NF-κB and that tetrandrine exhibits an inhibitory effect on activation of NF-κB. Although reactive oxygen species have been considered to be involved in the mechanism of PMA-induced NF-κB activation, most studies have been focused on H_2O_2 [12,16], with limited studies on O_2^- [14]. It may be noted that *N*-acetyl-L-cysteine has frequently been used as an oxidant to study the role of reactive oxygen species [13]. Although *N*-acetyl-L-cysteine can scavenge •OH radical, it increases the intracellular level of glutathione, which serves as a co-substance to eliminate H_2O_2. Thus the inhibitory effect of *N*-acetyl-L-cysteine did not necessarily imply the role of •OH radical in the mechanism of the NF-κB activation. It is known that H_2O_2 is able to be converted to very reactive •OH radicals, especially in the presence of metal ions [13]. The results obtained from the present study show H_2O_2 is indeed involved in the PMA-induced NF-κB activation in agreement with earlier studies [12,13]. SOD, whose function is the dismutation of O_2^- and the generation of H_2O_2 and molecular oxygen (Equation [a]), enhanced the activation in accordance with an earlier report [14].

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad [a]$$

More importantly, the results demonstrate that the •OH radical scavenger, formate, inhibited NF-κB activation by PMA, showing that •OH radicals play an important role in the mechanism of NF-κB activation. The role of •OH radical was further supported by the inhibition of deferoxamine. Deferoxamine is a chelator blocking the metal-catalyzed •OH generation from H_2O_2 [22,23]. For example, deferoxamine blocked Fe-mediated Haber-

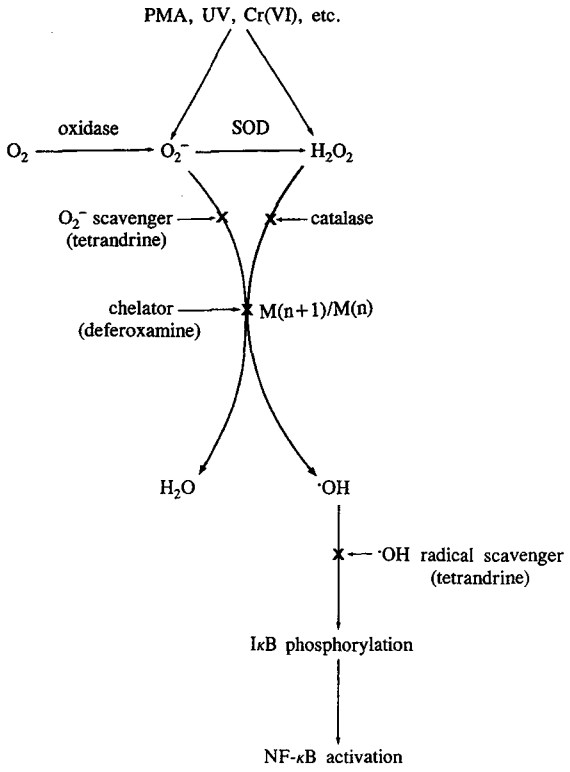

Fig. 6. Model of NF-κB activation.

Weiss reactions as shown in the following reactions (Equations [b]– [d]) [24].

$$O_2^- + Fe(III) \longrightarrow Fe(II) + O_2 \quad [b]$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + \bullet OH + OH^- \quad [c]$$

Overall,

$$O_2^- + H_2O_2 \xrightarrow{Fe(III)/Fe(II)} H_2O + \bullet OH + OH^- \quad [d]$$

Since Fe functions as a catalyst, a trace of Fe will generate a significant amount of •OH radicals from H_2O_2.

The present study has also evaluated the antioxidant property of tetrandrine. The results obtained show that tetrandrine is capable of scavenging •OH radical with a reaction rate constant of 1.4 x $10^{10} M^{-1}sec^{-1}$. It may be noted that the concentration of tetrandrine used in this study is high. Since •OH radical is very reactive, a trapping agent has been used for its detection. The spin trap utilized in this study, DMPO, is 75 mM, a typical amount for its trapping efficiency. Because the spin trapping agent itself is an excellent •OH scavenger, DMPO will react with •OH radical in competition with tetrandrine. This competition reaction requires a relatively high concentration of tetrandrine. Since the concentrations of DMPO and tetrandrine used are comparable, and addition of tetrandrine reduced the DMPO/•OH signal intensity by several fold, it can be concluded that tetrandrine is a good •OH radical scavenger. Similarly, the results obtained from the present study show that tetrandrine also scavenges O_2^- radical. It may be noted that SOD is frequently used to study the role of O_2^-. As mentioned in the previous section, SOD very efficiently dismutases O_2^- to generate H_2O_2 and molecular oxygen. The H_2O_2 generated can participate in the further reaction, ie, causing NF-κB activation [14]. In contrast, when tetrandrine scavenged O_2^-, H_2O_2 was not generated. The free-radical scavenging property may account for the overall inhibition of tetrandrine on PMA-induced NF-κB activation, although an alternative mechanism may exist. A model for NF-κB activation is presented in Fig. 6.

It is known that NF-κB plays a critical role in cells of the immune system. It is rapidly activated by a variety of pathogenic signals and is a potent transcriptional activator [10,11]. Since NF-κB is involved in many activities that tetrandrine can block, for example, anti-inflammatory and immunosuppressive actions, the inhibition of NF-κB activation by tetrandrine may account for tetrandrine's pharmacological actions.

The present results lead to the following conclusions: (a) •OH radicals play an important role in the mechanism of NF-κB activation induced by PMA; (b) tetrandrine inhibits the PMA-induced NF-κB activation; and (c) the free-radical scavenging property of tetrandrine may account for its inhibitory effect. Because of its capability to modulate NF-κB activation, tetrandrine has a potential use in modulating the expression of genes regulated by NF-κB.

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