

Sanguinarine (Pseudochelerythrine) Is a Potent Inhibitor of NF- κ B Activation, I κ B α Phosphorylation, and Degradation*

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The nuclear factor NF- κ B is a pleiotropic transcription factor whose activation results in inflammation, viral replication, and growth modulation. Due to its role in pathogenesis, NF- κ B is considered a key target for drug development. In the present report we show that sanguinarine (a benzophenanthridine alkaloid), a known anti-inflammatory agent, is a potent inhibitor of NF- κ B activation. Treatment of human myeloid ML-1a cells with tumor necrosis factor rapidly activated NF- κ B, this activation was completely suppressed by sanguinarine in a dose- and time-dependent manner. Sanguinarine did not inhibit the binding of NF- κ B protein to the DNA but rather inhibited the pathway leading to NF- κ B activation. The reversal of inhibitory effects of sanguinarine by reducing agents suggests a critical sulfhydryl group is involved in NF- κ B activation. Sanguinarine blocked the tumor necrosis factor-induced phosphorylation and degradation of I κ B α , an inhibitory subunit of NF- κ B, and inhibited translocation of p65 subunit to the nucleus. As sanguinarine also inhibited NF- κ B activation induced by interleukin-1, phorbol ester, and okadaic acid but not that activated by hydrogen peroxide or ceramide, the pathway leading to NF- κ B activation is likely different for different inducers. Overall, our results demonstrate that sanguinarine is a potent suppressor of NF- κ B activation and it acts at a step prior to I κ B α phosphorylation.

Sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) is derived from the root of *Sanguinaria canadensis* and other poppy-fumaria species (for references, see Ref. 1). This benzophenanthridine alkaloid is a structural homologue of chelerythrine, which is a potent inhibitor of protein kinase C (2). Sanguinarine has been shown to display antitumor (3) and anti-inflammatory properties in animals (4) and to inhibit neutrophil function, including degranulation and phagocytosis *in vitro* (5). It is also a potent inhibitor of Na-K-dependent ATPase (6–8) and cholinesterase (9).

NF- κ B is a nuclear transcription factor that resides in its inactive state in the cytoplasm as a heterotrimer consisting of p50, p65, and I κ B α subunits. On activation of the complex, I κ B α sequentially undergoes phosphorylation, ubiquitination, and degradation, thus releasing the p50-p65 heterodimer for

translocation to the nucleus (for references, see Ref. 10). Treatment of cells with various inflammatory and stress stimuli including lipopolysaccharide, tumor necrosis factor (TNF),¹ interleukin (IL)-1, ceramide, hydrogen peroxides, ultraviolet light, phorbol myristate acetate (PMA), and okadaic acid (OA) activates the transcription factor. NF- κ B is a ubiquitous transcription factor that regulates the expression of various cytokines, their receptors, major histocompatibility complex genes, cell adhesion proteins, and other gene products involved in inflammation, septic shock, atherosclerosis, cell proliferation, and viral replication including human immunodeficiency virus-1.

Sanguinarine exhibits antimicrobial, anti-inflammatory, and antioxidant properties (5, 11–17). Since anti-inflammatory compounds like aspirin and glucocorticoid have been shown to inhibit activation of NF- κ B (18–20), we investigated the effect of sanguinarine on activation of NF- κ B induced by a wide variety of agents including TNF. The results show that sanguinarine is a potent inhibitor of NF- κ B activation that blocks the phosphorylation and degradation of I κ B α , thus suggesting that sanguinarine is a potential candidate for intervening in NF- κ B-dependent pathological responses.

EXPERIMENTAL PROCEDURES

Materials—A highly purified preparation of sanguinarine was kindly provided by Dr. Ken Godowski of ATRIX Laboratories Inc. (Fort Collins, CO). OA was obtained from LC Laboratories (Woburn, MA). Ouabain and chelerythrine chloride were obtained from Research Biochemical International (Natick, MA). Ceramide (C2) was obtained from Calbiochem (San Diego, CA). Berberine, bovine serum albumin, and PMA were obtained from Sigma. [γ -³²P]ATP with a specific activity of 7000 Ci/mmol was obtained from ICN (Costa Mesa, CA). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, and IL-1 were kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum were obtained from Life Technologies, Inc. (Grand Island, NY). Antibodies against I κ B α , cyclin D1, the NF- κ B subunits p50 and p65, and c-Rel were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines—Histiocytic lymphoma (U937), myeloid (ML-1a), normal human foreskin fibroblast (FS4), and T (Jurkat) cells were routinely grown in RPMI 1640 medium supplemented with glutamine (2 mM), gentamicin (50 μ g/ml), and fetal bovine serum (10%). The cells were seeded at a density of 1×10^5 cells/ml in T25 flasks (Falcon 3013, Becton Dickinson Labware, Lincoln Park, NJ) containing 10 ml of medium and grown at 37 °C in an atmosphere of 95% air and 5% CO₂. Cultures were split when cell density reached $1-2 \times 10^6$ /ml.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were carried out as previously described in detail (21, 22). Briefly, nuclear extracts (NE) were prepared from 2×10^6 cells after different treatments and

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¹ The abbreviations used are: TNF, tumor necrosis factor; IL-1, interleukin-1; PMA, phorbol myristate acetate; OA, okadaic acid; DTT, dithiothreitol; NE, nuclear extracts; EMSA, electrophoretic mobility shift assay; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

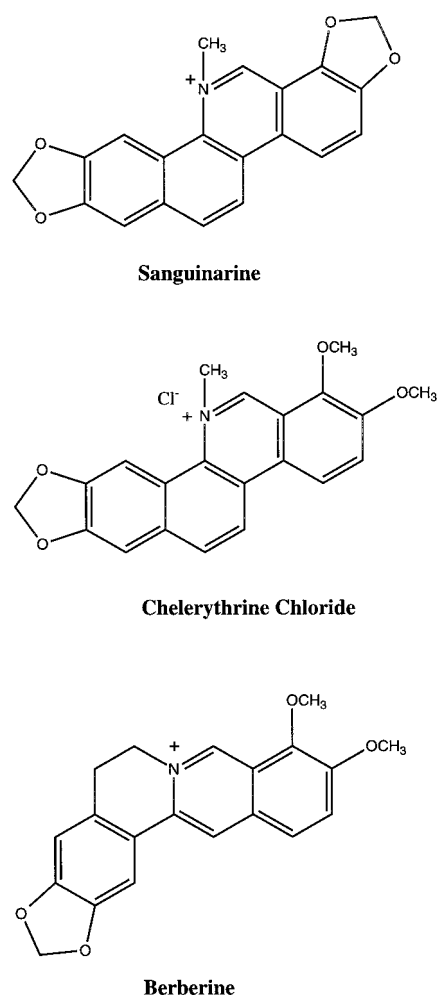


FIG. 1. Structural similarity between sanguinarine, chelerythrine chloride, and berberine.

then either used immediately or stored at -70°C . EMSA was performed by incubating $4\text{ }\mu\text{g}$ of NE for 15 min at 37°C with 8 fmol of ^{32}P -end-labeled 45-mer double-stranded oligonucleotide containing the NF- κ B-binding site (5'-TTGTTACAAGGGACTTTCCGCTGGGGACT-TTCAGGGAGGCGTGG-3') from the human immunodeficiency virus long terminal repeat. The DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels, and the gel was then dried. A double-stranded oligonucleotide with mutated NF- κ B sites (5'-TTGTTACAAGTCACTTTCCGCTGCTCACTTTCCAGGGA-GGCGTGG-3') was used to examine the specificity of binding of NF- κ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, NE prepared from TNF-treated cells were incubated with the antibodies against either p50 or p65 subunits of NF- κ B for 30 min at 37°C before the complex was analyzed by EMSA. Antibody against cyclin D1 was included as a negative control.

Western Blotting for I κ B α and p65—Western blotting was carried out essentially as described previously (23). Briefly, the cytoplasmic extracts from treated cells were resolved on 10% SDS-polyacrylamide gels for I κ B α . To determine p65 levels, NE and cytoplasmic extracts were resolved on 9% SDS-polyacrylamide gels, electrotransferred to Immobilon P membranes, probed with a rabbit polyclonal antibody against p65, and detected by chemiluminescence (ECL-Amersham).

RESULTS

The structures of sanguinarine and its analogues are shown in Fig. 1. Chelerythrine, a near homologue of sanguinarine, is a specific inhibitor of protein kinase C (2). Both berberine and sanguinarine interact with DNA (24). Our tests of these agents showed that, under the conditions we used, they had no effect on cell viability as determined by trypan blue exclusion (data not shown).

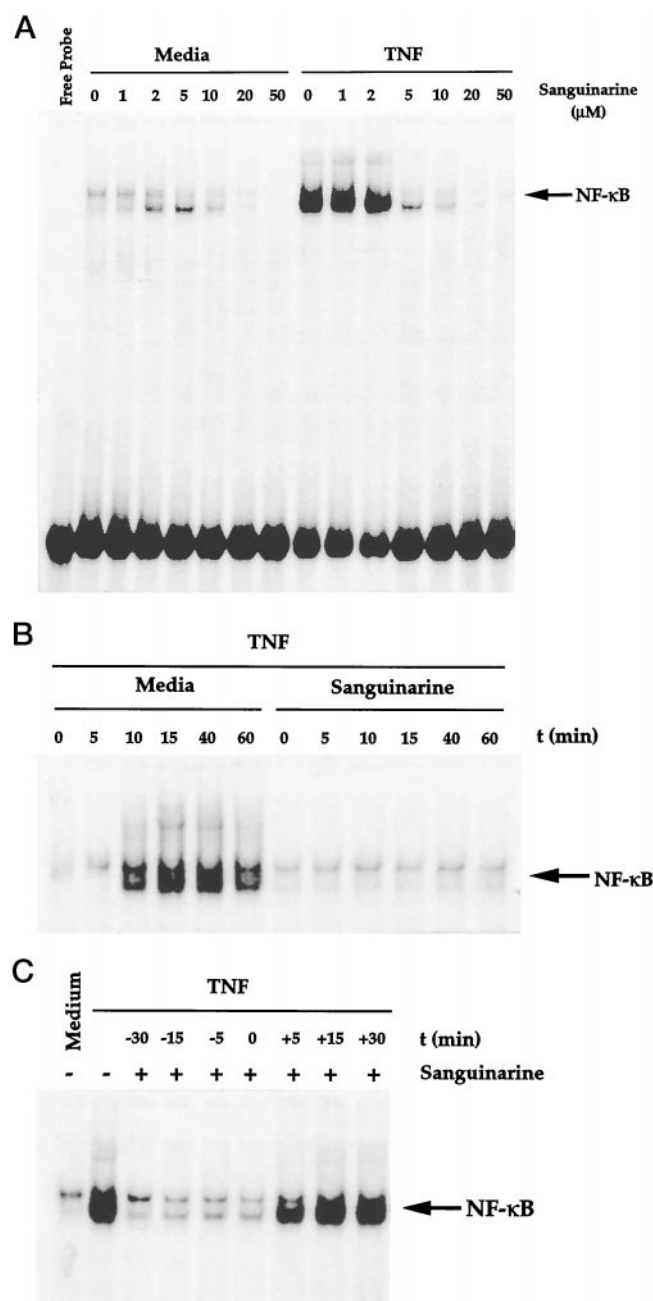


FIG. 2. A, dose-response to sanguinarine for the inhibition of TNF induced NF- κ B activation. ML-1a cells ($2 \times 10^6/\text{ml}$) were preincubated for 30 min at 37°C with the indicated concentrations of sanguinarine and then for 30 min with or without 10 pM TNF. Nuclear extracts were prepared and NF- κ B was assayed by EMSA as described under "Experimental Procedures." B, effect of sanguinarine on the kinetics of TNF-induced NF- κ B activation. ML-1a cells ($2 \times 10^6/\text{ml}$) were preincubated for 30 min at 37°C with or without $5\text{ }\mu\text{M}$ sanguinarine and then treated with TNF (10 pM) for the indicated time periods. Nuclear extracts were prepared and NF- κ B was assayed as described. C, effect of pre-, co-, and postincubation of cells with sanguinarine on TNF-induced NF- κ B. ML-1a cells ($2 \times 10^6/\text{ml}$) were incubated at 37°C with $5\text{ }\mu\text{M}$ sanguinarine at the times indicated and then tested for activation of NF- κ B in the presence of 10 pM TNF for 30 min. -, sanguinarine present before the addition of TNF; 0, sanguinarine coincubated with TNF; +, sanguinarine added after TNF.

Sanguinarine Inhibits TNF-dependent Activation of NF- κ B—ML-1a cells were pretreated with different concentrations of sanguinarine (up to $50\text{ }\mu\text{M}$) for 30 min, incubated either with or without TNF (10 pM) for 30 min at 37°C , and then examined for NF- κ B activation by EMSA. Fig. 2A shows that sanguinarine inhibited the TNF-dependent activation of NF- κ B in a

dose-dependent manner, with maximum effect occurring at 10 μ M. The IC_{50} of sanguinarine for the inhibition of TNF-dependent NF- κ B was calculated to be approximately 3 μ M (data not shown). Sanguinarine by itself did not activate NF- κ B at 1, 10, 20, and 50 μ M concentrations; however, a very low but reproducible activation of NF- κ B was observed at 2 and 5 μ M (Fig. 2A). TNF activated NF- κ B within 10 min and reached maximum at 15–40 min (Fig. 2B). No activation of NF- κ B by TNF was observed in cells pretreated with sanguinarine.

To gain further insight into the kinetics of inhibition, we preincubated the cells with sanguinarine for 30, 15, and 5 min and then exposed them to TNF. Sanguinarine was also added at the same time as 0 min and 5, 15, and 30 min after TNF. In every case, TNF was present for 30 min. As shown in Fig. 2C, coinubation of cells with sanguinarine and TNF was as effective as preincubation in blocking the activation of NF- κ B. Sanguinarine could also block, although only partially, the NF- κ B activation even when added 5 min after TNF. It had no effect when added 10 min or later after TNF.

Previous studies from our laboratory have shown that a high concentration of TNF (10 nM) induces more robust and rapid (within 5 min) activation of NF- κ B (22). To determine whether sanguinarine could also inhibit a robust response to TNF, sanguinarine-pretreated cells were challenged with increasing concentrations of TNF (up to 10 nM) for 30 min and then examined for NF- κ B (Fig. 3A). Although the activation of NF- κ B by 10 nM TNF was very strong, sanguinarine inhibited it just as effectively as it did the 0.01 nM concentration. These results show that sanguinarine is a very potent inhibitor of NF- κ B activation.

Since NF- κ B is a family of proteins, various combinations of Rel/NF- κ B protein can constitute an active NF- κ B heterodimer that binds to a specific sequence in DNA (10). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF- κ B, we incubated NE from TNF-activated cells with antibody to either p50 (NF- κ B1) or p65 (Rel A) subunits and then conducted EMSA. Antibodies to either subunit shifted the band completely to a higher molecular weight (Fig. 3B). Antibody against c-Rel or unrelated antibody, like antibody to cyclin D1, did not shift the NF- κ B band, thus suggesting that the TNF-activated complex consists of p50 and p65 subunits. The activation of NF- κ B by TNF, as indicated by the band on EMSA, was quite specific since the band disappeared when unlabeled oligo was added. Also no binding took place when we used a mutant oligonucleotide in which CTC was substituted for GGG at the NF- κ B-binding site (Fig. 3B).

Sanguinarine Does Not Interfere with the Binding of NF- κ B to DNA—It has been shown that both TPCK, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, down-regulate NF- κ B by chemical modification of the NF- κ B subunits, thus preventing its binding to DNA (25, 26). To determine whether sanguinarine also modifies NF- κ B proteins, we incubated NE prepared from TNF-activated cells with different concentrations of sanguinarine *in vitro*. EMSA (Fig. 3C) showed that sanguinarine at a concentration as high as 100 μ M did not modify the ability of NF- κ B to bind to the DNA.

Inhibition of NF- κ B Activation by Sanguinarine Is Not Cell Type Specific—We also examined the ability of sanguinarine to block TNF-induced NF- κ B activation in another myeloid line (U937) and in lymphoid (Jurkat) and diploid fibroblast (FS4) cells. The results of these experiments (Fig. 4) indicate that sanguinarine inhibited NF- κ B in all three cell types. Almost complete inhibition was observed with 5 μ M sanguinarine, thus suggesting that this effect of sanguinarine is not cell type specific. Interestingly, a strong constitutive band was observed in Jurkat cells that bound specifically to the NF- κ B site, as is

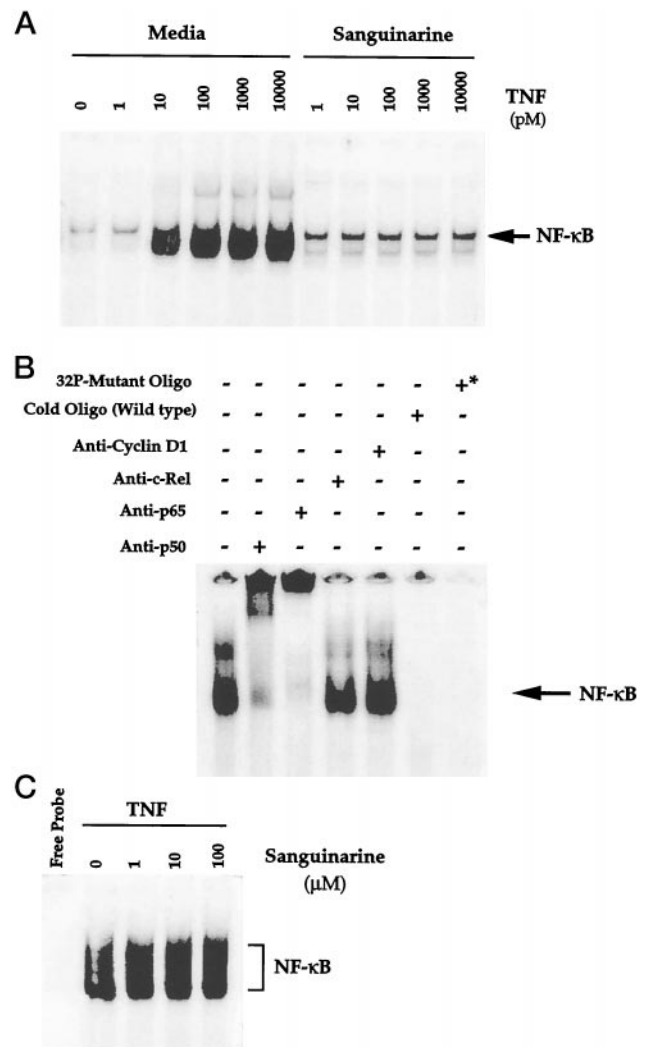


FIG. 3. A, effect of sanguinarine on NF- κ B activation induced by high doses of TNF. ML-1a cells (2×10^6 /ml) were preincubated for 30 min at 37 °C with or without 5 μ M sanguinarine and then treated for 30 min with the indicated concentrations of TNF. Nuclear extracts were prepared and NF- κ B was assayed. B, supershift and specificity of TNF-induced NF- κ B activation. Nuclear extracts were prepared from TNF (10 pM)-activated ML-1a cells, incubated for 15 min with antibodies or cold wild-type NF- κ B oligonucleotides (25-fold molar excess), and then assayed for NF- κ B as described. *, EMSA was performed in the presence of a 32 P-labeled mutant oligonucleotide in which the three Gs in the NF- κ B-binding site were mutated to CTC. C, effect of sanguinarine on the binding of NF- κ B to DNA. Nuclear extracts prepared from TNF (10 pM)-activated ML-1a cells were incubated for 20 min with indicated concentrations of sanguinarine and analyzed for NF- κ B activation.

shown by the abrogation of binding by a 25-fold molar excess of unlabeled oligonucleotide. Formation of this band was not inhibited by sanguinarine, suggesting that sanguinarine inhibited only the TNF-inducible form of NF- κ B (p50-p65 heterodimer). The composition of this constitutive band in Jurkat cells as determined by supershift analysis was found to be entirely p50 homodimer (data not shown).

Inhibition of TNF-mediated NF- κ B Activation by Sanguinarine Is Not through Inhibition of Na/K-ATPase—Several reports indicate that sanguinarine can inhibit Na/K-ATPase (6–8). To determine if sanguinarine blocks NF- κ B activation by inhibiting this ATPase, we tested the ability of ouabain, a well known, potent inhibitor of Na/K-ATPase (27, 28), to block TNF-induced NF- κ B activation. Ouabain had no effect on TNF-induced activation of this transcription factor (Fig. 5, upper panel). Thus it appears that sanguinarine blocks NF- κ B activation through some other mechanism.

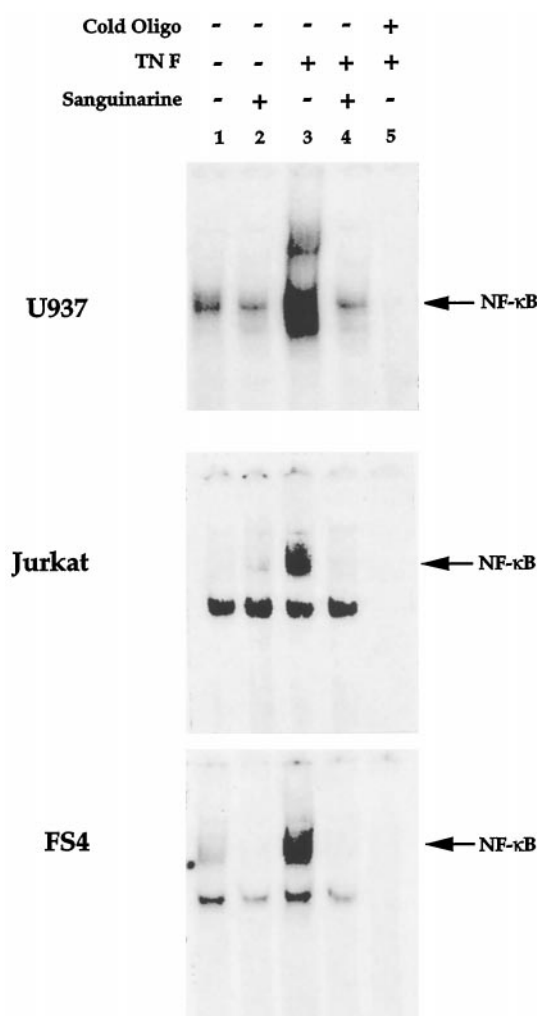


FIG. 4. Effect of sanguinarine on TNF-induced NF- κ B in different cell types. U937, Jurkat, or FS4 cells (2×10^6 /ml) were preincubated for 30 min with or without $5 \mu\text{M}$ sanguinarine, followed by TNF (10 pM) for 30 min. Nuclear extracts were prepared and tested for NF- κ B activation as described under "Experimental Procedures." Specificity of NF- κ B binding was determined by performing EMSA in the presence of 25-fold molar excess of cold NF- κ B oligonucleotide.

Structural Analogues of Sanguinarine Do Not Inhibit NF- κ B Activation—As shown in Fig. 1, chelerythrine and berberine have a strong structural similarity to sanguinarine. Therefore, we investigated their ability to block NF- κ B activation. As shown in Fig. 5, neither chelerythrine (*middle panel*) nor berberine (*lower panel*) had any effect on TNF-induced NF- κ B activation. As chelerythrine is known to inhibit protein kinase C (2) and berberine mediates its effects by binding to DNA (24), the results indicate that sanguinarine does not block NF- κ B activation by either of these mechanisms.

Sanguinarine Blocks the NF- κ B Activation by OA, PMA, Ceramide, H_2O_2 , and IL-1—Besides TNF, NF- κ B is also activated by wide variety of other agents including OA, PMA, ceramide-C2, H_2O_2 , and IL-1. However, whether activation by these agents occurs by the same mechanism as that by TNF is not clear. Therefore, we examined the ability of sanguinarine to block NF- κ B activation induced by different agents. As shown in Fig. 6, sanguinarine completely blocked the activation of NF- κ B by PMA, IL-1, and OA. Interestingly, it only partially blocked the effect of ceramide and had no effect on H_2O_2 -induced NF- κ B activation. These results suggest that the pathway leading to NF- κ B activation by TNF, PMA, IL-1, and OA is different from that induced by ceramide and H_2O_2 .

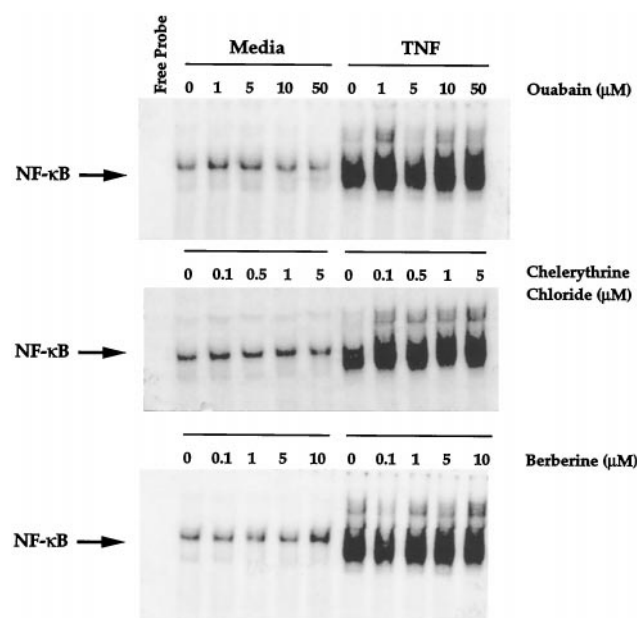


FIG. 5. Effect of different concentrations of ouabain, chelerythrine chloride, and berberine on TNF-mediated activation of NF- κ B. U937 cells (2×10^6 /ml) were preincubated for 60 min at 37°C with ouabain, chelerythrine, and berberine followed by treatments at 37°C with TNF (100 pM) for 30 min and then tested for NF- κ B activation as described under "Experimental Procedures."

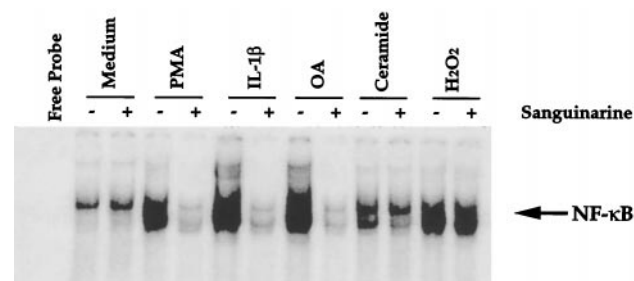


FIG. 6. Effect of sanguinarine on PMA-, IL-1-, ceramide-, okadaic acid- and H_2O_2 -mediated activation of NF- κ B. U937 cells (2×10^6 /ml) were preincubated for 60 min at 37°C with sanguinarine ($5 \mu\text{M}$) followed by treatments at 37°C for 60 min with PMA (100 ng/ml), IL-1 (200 ng/ml), H_2O_2 (1 mM), or ceramide-C2 ($10 \mu\text{M}$) or okadaic acid (600 nM) and then tested for NF- κ B activation as described under "Experimental Procedures."

The Reducing Agent DTT Reverses the Inhibitory Effect of Sanguinarine—The biological effects of phenylarsine oxide, *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), herbimycin A, and caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester on suppression of NF- κ B activation can be reversed by reducing agents (25, 26, 29, 30). Also, sanguinarine contains an iminium ion that can form a pseudobase with thiol groups of the proteins (31). Therefore, we examined the ability of DTT to reverse the inhibitory effect of sanguinarine on TNF-induced NF- κ B activation. Cells were treated with sanguinarine in the presence and absence of DTT and then examined for the activation of NF- κ B by TNF. As shown in Fig. 7, DTT by itself did not have any effect on TNF-dependent activation of NF- κ B, but it completely reversed the inhibition induced by sanguinarine. These results demonstrate the critical role of sulfhydryl groups in the TNF-dependent activation of NF- κ B.

Sanguinarine contains a thiol-reactive iminium group, and if the thiol groups of NF- κ B were already blocked by DTT in the NE and EMSA buffers, sanguinarine could not have modified the NF- κ B proteins *in vitro*. However, the results remained the same when the NE were prepared in DTT-free buffer and when

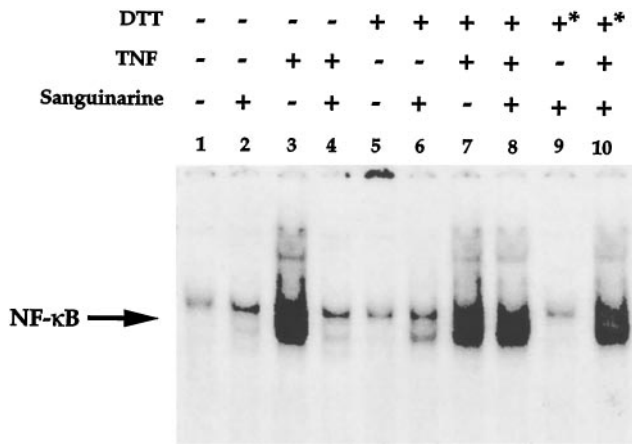


FIG. 7. Effect of DTT on sanguinarine-induced inhibition of NF- κ B activation. ML-1a cells (2×10^6 /ml) were incubated for 30 min with DTT (100 μ M), and then with or without sanguinarine (5 μ M) for 30 min, and then activated with TNF (100 pM) for 30 min. Nuclear extracts were prepared and assayed for NF- κ B as described. In the sets marked with *, DTT was added along with sanguinarine for 30 min.

the DTT was omitted from EMSA buffer (data not shown). Therefore, sanguinarine inhibits NF- κ B activation through a mechanism different from that of TPCK or herbimycin A.

Sanguinarine Inhibits TNF-dependent Phosphorylation and Degradation of I κ B α —The translocation of NF- κ B to the nucleus is preceded by the phosphorylation and proteolytic degradation of I κ B α (for review, see Ref. 10). To determine whether sanguinarine affected I κ B α degradation, the kinetics of activation of NF- κ B by TNF under the influence of sanguinarine was studied. Nuclear and cytoplasmic extracts were prepared for EMSA and I κ B α Western blot analysis, respectively. Activation of NF- κ B by 10 pM TNF occurred as early as 10 min of treatment, peaked between 15 and 40 min, and declined by 60 min (Fig. 2B). Pretreatment with sanguinarine inhibited NF- κ B activation at all the time points of TNF treatment. Western blot analysis of I κ B α protein in cytoplasmic extracts revealed that TNF treatment of cells caused a slower migrating band of I κ B α to appear within 5 min; by 15 min I κ B α mostly disappeared, and by 60 min it reappeared (Fig. 8A). The disappearance and reappearance of I κ B α corresponded with the peak and decline of NF- κ B, respectively. Pretreatment of cells with sanguinarine abolished the appearance of the TNF-induced slower migrating band, and the degradation of I κ B α . The appearance of the slower migrating band has been shown to be induced by phosphorylation of I κ B α at serine 32 and 36 (25, 32).

Because NF- κ B activation also requires nuclear translocation of the p65 subunit of NF- κ B, we examined the cytoplasmic and nuclear pool of p65 protein by Western blot analysis. As shown in Fig. 8B, the TNF-induced appearance of p65 in the nucleus was blocked by sanguinarine. These results indicate that sanguinarine prevented phosphorylation and degradation of I κ B α , and hence the nuclear translocation of NF- κ B.

DISCUSSION

In our study, sanguinarine completely blocked the activation of NF- κ B induced by TNF, IL-1, phorbol ester, and OA but not that activated by H₂O₂ and ceramide (Fig. 9). Furthermore, the effect was not cell type specific. Chelerythrine and berberine, structural analogues of sanguinarine, had no effect on NF- κ B activation. Sanguinarine's inhibitory effects were not due to inhibition of Na⁺/K⁺-ATPase. Rather, sanguinarine blocked the phosphorylation and degradation of I κ B α an inhibitory subunit of NF- κ B.

How sanguinarine inhibits the activation of NF- κ B is not

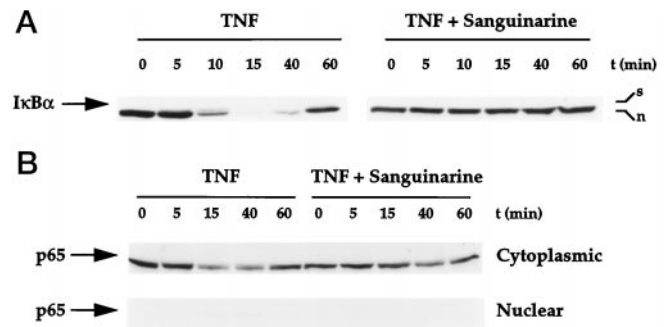


FIG. 8. Effect of sanguinarine on the kinetics of TNF-induced I κ B α degradation and the level of p65 in cytoplasm and nucleus. A, ML-1a cells (2×10^6 /ml) were preincubated for 30 min at 37°C with or without 5 μ M sanguinarine and then treated with TNF (10 pM) for the indicated times. Nuclear extracts were prepared and NF- κ B was assayed (see Fig. 2B) and the cytoplasmic extracts were prepared from the cells and assayed for I κ B α by Western blot analysis as described. B, cells were preincubated for 30 min at 37°C with or without 5 μ M sanguinarine and then treated with TNF (10 pM) for the indicated time periods. Cytoplasmic and nuclear extracts were prepared and assayed for p65 by Western blot analysis.

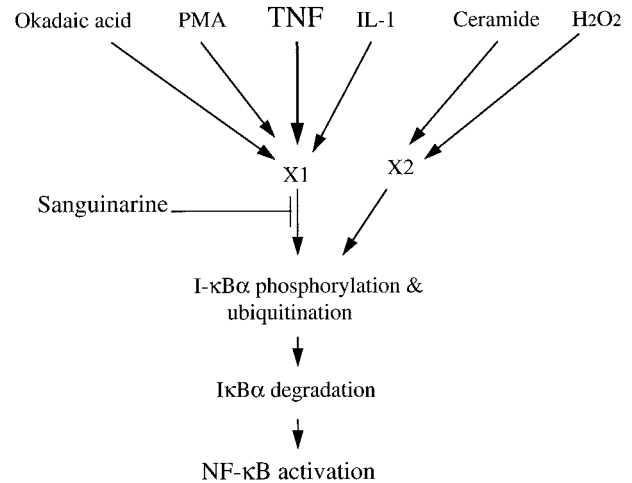


FIG. 9. Site of action of sanguinarine in the pathway leading to NF- κ B activation by a variety of agents.

clear at present. It enters the cells rapidly without altering membrane fluidity and localizes in the nucleus (5). In the nucleus it intercalates with DNA, preferring GC-rich sequences (24, 33, 34) but this is not how it inhibits NF- κ B activation in as much as *in vitro* sanguinarine treatment did not inhibit binding of NF- κ B to the DNA. Likewise, berberine, which is also known to intercalate into DNA (24), had no effect on NF- κ B activation. Nor did sanguinarine directly chemically modify NF- κ B. In contrast, herbimycin A and TPCK (25, 26), both of which inhibit NF- κ B activation, can modulate the RXXRXXXC sequence present in NF- κ B proteins (25, 35).

Several reports indicate that sanguinarine inhibits a Na/K-ATPase (6–8). Since ouabain, a potent Na/K-ATPase inhibitor had no effect on TNF-induced NF- κ B activation, sanguinarine must not inhibit NF- κ B activation by blocking this ATPase.

Our results show that sanguinarine inhibits NF- κ B activation by inhibiting the phosphorylation and degradation of I κ B α , the inhibitory subunit of NF- κ B. Two observations also suggest that sanguinarine targets an early step in the activation of NF- κ B, the most likely being either I κ B kinase or a step that modulates I κ B kinase. 1) It inhibits NF- κ B when pre- or co-incubated with TNF, but not when added 10 min after the addition of TNF. 2) It inhibits only inducible NF- κ B, and not the constitutive p50 homodimer. Also, it inhibits activation of

NF- κ B in human foreskin fibroblast cells, FS4, where inducible NF- κ B is known to be regulated via I κ B β (46). The activation of inducible NF- κ B requires phosphorylation of I κ B α at serine 32 and 36 and of I κ B β at serine 19 and 23 (32). Both I κ Bs have been postulated to be phosphorylated by either the same kinase or by similar kinases (32). The phosphorylation of I κ Bs results in ubiquitination at lysines 21 and 22 in I κ B α and lysine 22 in I κ B β (32). Polyubiquitinated I κ Bs are targeted to proteasomes and quickly degraded (37). Inhibition of phosphorylation results in inhibition of I κ B degradation. Indeed, sanguinarine inhibits inducible phosphorylation of I κ B α and its subsequent degradation.

How protein kinase is activated by the inducers of NF- κ B activation is not certain, but the production of reactive oxygen intermediates upstream from protein kinase has been suggested (38, 39). In human peripheral blood neutrophils, sanguinarine has been shown to inhibit PMA-induced oxidative bursts and generation of superoxide anions (5, 17). Also, sanguinarine has been shown to have antioxidative activity against spontaneous oxidation (40). Therefore, it is possible that sanguinarine suppresses NF- κ B activation by inhibiting reactive oxygen intermediate generation. This is consistent with our findings that sanguinarine inhibited, besides TNF, NF- κ B activation induced by PMA, okadaic acid, and IL-1. Interestingly, however, sanguinarine did not block ceramide or H₂O₂-induced NF- κ B activation. These results suggest a difference in the signaling pathway leading to NF- κ B activation by different agents.

Recently, the putative I κ B α kinase has been shown to be regulated by ubiquitination (41). Whether sanguinarine can directly interfere with any of these processes is not known. However, sanguinarine has a reactive iminium group that has been shown to form pseudobases with thiol groups of several proteins (31, 42–45), including some kinases (42); it is likely that it modifies some reactive thiol groups of some protein(s) required for the phosphorylation of I κ B. Reducing agents like DTT form a complex with the iminium ion of sanguinarine, thus neutralizing its thiol-reactive properties (42). Since pretreatment of cells with DTT and incubation of DTT and sanguinarine abolished the inhibitory effect of sanguinarine on TNF-induced NF- κ B activation, it is possible that sanguinarine acts by directly modifying either I κ B kinase or some other intermediate upstream to the kinase. These intermediates must be common to the activation pathway of a number of inflammatory agents tested here (Fig. 9). Overall our results presented provides a novel mechanism by which sanguinarine may exhibit its anti-inflammatory effects.

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