Sanguinarine (Pseudochelerythrine) Is a Potent Inhibitor of NF-κB Activation, IκBα Phosphorylation, and Degradation*

(Received for publication, August 15, 1997, and in revised form, September 19, 1997)

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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-κB is a nuclear transcription factor that resides in its inactive state in the cytoplasm as a heterotrimer consisting of p50, p65, and c-Rel. On activation of the complex, IκBα 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), interleukin (IL)-1, ceramide, hydrogen peroxides, ultraviolet light, phorbol myristate acetate (PMA), and okadaic acid (OA) activates the transcription factor. NF-κB 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-κB (18–20), we investigated the effect of sanguinarine on activation of NF-κB induced by a wide variety of agents including TNF. The results show that sanguinarine is a potent inhibitor of NF-κB activation that blocks the phosphorylation and degradation of IκBα, thus suggesting that sanguinarine is a potential candidate for intervening in NF-κB-dependent pathological responses.

EXPERIMENTAL PROCEDURES

Materials—A highly purified preparation of sanguinarine was kindly provided by Dr. Ken Godowski of ATRIX Laboratories Inc. (Port 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 × 107 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 IκBα, cyclin D1, the NF-κB 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 μg/ml), and fetal bovine serum (10%). The cells were seeded at a density of 1 × 10⁵ 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 × 10⁶/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 × 10⁶ cells after different treatments and

* This research was supported by a grant from The Clayton Foundation for Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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, t-1-tosylamido-2-phenylethyl chloromethyl ketone.
then either used immediately or stored at -70 °C. EMSA was performed by incubating 4 μg of NE for 15 min at 37 °C with 8 fmol of 32P-end-labeled 45-mer double-stranded oligonucleotide containing the NF-κB-binding site (5'-TTGTTAACGGGACTTTCCGCTGGGGACTTTCCAGGAGGGCTG-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-κB sites (5'-TTGTTAACCTCACTTTCGCTGCTCACTTTCCAGGAGGGCTG-3') was used to examine the specificity of binding of NF-κB 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-κB 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 IkBa 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 IkBa. To determine p65 levels, NE and cytoplasmic extracts were resolved on 9% SDS-polyacrylamide gels, electrophoresed 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).

Sanguinarine Inhibits TNF-dependent Activation of NF-κB—ML-1a cells were pretreated with different concentrations of sanguinarine (up to 50 μM) for 30 min, incubated either with or without 10 pM TNF. Nuclear extracts were prepared and NF-κB was assayed by EMSA. Then for 30 min with or without 10 pM TNF. Nuclear extracts were prepared and NF-κB was assayed by EMSA as described under "Experimental Procedures." B, effect of sanguinarine on the kinetics of TNF-induced NF-κB activation. ML-1a cells (2 × 10⁴/ml) were preincubated for 30 min at 37°C with or without 5 μM sanguinarine and then treated with TNF (10 pM) for the indicated time periods. Nuclear extracts were prepared and NF-κB was assayed as described. C, effect of pre-, co-, and postincubation of cells with sanguinarine on TNF-induced NF-κB. ML-1a cells (2 × 10⁴/ml) were incubated at 37°C with 5 μM sanguinarine at the times indicated and then tested for activation of NF-κB in the presence of 10 pM TNF for 30 min. -, sanguinarine added before the addition of TNF; 0, sanguinarine coincubated with TNF; +, sanguinarine added after TNF.
Results show that sanguinarine is a very potent inhibitor of NF-κB activation.

Since NF-κB is a family of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB 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-κB, we incubated NE from TNF-activated cells with antibody to either p50 (NF-κB1) or p65 (Rel A) subunits and then conducted EMSA. Antibodies to either subunit shifted the band completely to a higher molecular weight (Fig. 3A). Antibody against c-Rel or unrelated antibody, like antibody to cyclin D1, did not shift the NF-κB band, thus suggesting that the TNF-activated complex consists of p50 and p65 subunits. The activation of NF-κB 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-κB-binding site (Fig. 3B).

Sanguinarine Does Not Interfere with the Binding of NF-κB to DNA—It has been shown that both TPCK, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, down-regulate NF-κB by chemical modification of the NF-κB subunits, thus preventing its binding to DNA (25, 26). To determine whether sanguinarine also modifies NF-κB 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 μM did not modify the ability of NF-κB to bind to the DNA.

Inhibition of NF-κBActivation by Sanguinarine Is Not Cell Type Specific—We also examined the ability of sanguinarine to block TNF-induced NF-κB 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-κB in all three cell types. Almost complete inhibition was observed with 5 μM 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-κB site, as is 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-κB (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-κB 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-κB 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-κB activation. Ouabain had no effect on TNF-induced activation of this transcription factor (Fig. 5, upper panel). Thus it appears that sanguinarine blocks NF-κB activation through some other mechanism.
Structural Analogues of Sanguinarine Do Not Inhibit NF-κB Activation—As shown in Fig. 1, chelerythrine and berberine have a strong structural similarity to sanguinarine. Therefore, we investigated their ability to block NF-κB activation. As shown in Fig. 5, neither chelerythrine (middle panel) nor berberine (lower panel) had any effect on TNF-induced NF-κB 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-κB activation by either of these mechanisms.

Sanguinarine Blocks the NF-κB Activation by OA, PMA, Ceramide, H₂O₂, and IL-1—Besides TNF, NF-κB is also activated by wide variety of other agents including OA, PMA, ceramide-C₂, H₂O₂, 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-κB activation induced by different agents. As shown in Fig. 6, sanguinarine completely blocked the activation of NF-κB by PMA, IL-1, and OA. Interestingly, it only partially blocked the effect of ceramide and had no effect on H₂O₂-induced NF-κB activation. These results suggest that the pathway leading to NF-κB activation by TNF, PMA, IL-1, and OA is different from that induced by ceramide and H₂O₂.

The Reducing Agent DTT Reverses the Inhibitory Effect of Sanguinarine—The biological effects of phenylarsine oxide, N-tosyl-1-phenylalanyl chloromethyl ketone (TPCK), herbimycin A, and caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester on suppression of NF-κB 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-κB activation. Cells were treated with sanguinarine in the presence and absence of DTT and then tested for NF-κB activation as described under “Experimental Procedures.”

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

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**Fig. 4.** Effect of sanguinarine on TNF-induced NF-κB in different cell types. U937, Jurkat, or FS4 cells (2 × 10⁶/ml) were preincubated for 30 min with or without 5 μM sanguinarine, followed by TNF (10 pM) for 30 min. Nuclear extracts were prepared and tested for NF-κB activation as described under “Experimental Procedures.” Specificity of NF-κB binding was determined by performing EMSA in the presence of 25-fold molar excess of cold NF-κB oligonucleotide.

**Fig. 5.** Effect of different concentrations of ouabain, chelerythrine chloride, and berberine on TNF-mediated activation of NF-κB. U937 cells (2 × 10⁶/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-κB activation as described under “Experimental Procedures.”

**Fig. 6.** Effect of sanguinarine on PMA-, IL-1-, ceramide-, okadaic acid- and H₂O₂-mediated activation of NF-κB. U937 cells (2 × 10⁶/ml) were preincubated for 60 min at 37 °C with sanguinarine (5 μM), followed by treatments at 37 °C for 60 min with PMA (100 ng/ml), IL-1 (200 ng/ml), H₂O₂ (1 mM), or ceramide-C₂ (10 μM) or okadaic acid (600 nM) and then tested for NF-κB activation as described under “Experimental Procedures.”
the DTT was omitted from EMSA buffer (data not shown). Therefore, sanguinarine inhibits NF-κB activation through a mechanism different from that of TPCK or herbimycin A.

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

Because NF-κB activation also requires nuclear translocation of the p65 subunit of NF-κB, 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 IκBα, and hence the nuclear translocation of NF-κB.

DISCUSSION

In our study, sanguinarine completely blocked the activation of NF-κB 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-κB activation. Sanguinarine’s inhibitory effects were not due to inhibition of Na+/K+-ATPase. Rather, sanguinarine blocked the phosphorylation and degradation of IκBα an inhibitory subunit of NF-κB.

How sanguinarine inhibits the activation of NF-κB is not 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-κB activation in as much as in vitro sanguinarine treatment did not inhibit binding of NF-κB to the DNA. Likewise, berberine, which is also known to intercalate into DNA (24), had no effect on NF-κB activation. Nor did sanguinarine directly chemically modify NF-κB. In contrast, herbimycin A and TPCK (25, 26), both of which inhibit NF-κB activation, can modulate the RXXRXXXXC sequence present in NF-κB 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-κB activation, sanguinarine must not inhibit NF-κB activation by blocking this ATPase.

Our results show that sanguinarine inhibits NF-κB activation by inhibiting the phosphorylation and degradation of IκBα, the inhibitory subunit of NF-κB. Two observations also suggest that sanguinarine targets an early step in the activation of NF-κB, the most likely being either IκB kinase or a step that modulates IκB kinase. 1) It inhibits NF-κB when pre- or coincubated with TNF, but not when added 10 min after the addition of TNF. 2) It inhibits only inducible NF-κB, and not the constitutive p50 homodimer. Also, it inhibits activation of
NF-κB in human foreskin fibroblast cells, FS4, where inducible NF-κB is known to be regulated via IkBβ (46). The activation of inducible NF-κB requires phosphorylation of IkBα at serine 32 and 36 and of IkBβ at serine 19 and 23 (32). Both IkBs have been postulated to be phosphorylated by either the same kinase or by similar kinases (32). The phosphorylation of IkBs results in ubiquinitation at lysines 21 and 22 in IkBα and lysine 22 in IkBβ (32). Polyubiquinitated IkBs are targeted to proteasomes and quickly degraded (37). Inhibition of phosphorylation results in inhibition of IkB degradation. Indeed, sanguinarine inhibits inducible phosphorylation of IkBα and its subsequent degradation.

How protein kinase is activated by the inducers of NF-κB activation is not certain, but the production of reactive oxygen intermediates upstream from protein kinase has been postulated (32). The phosphorylation of IκBα and IκBβ results in inhibition of IκBα and quickly degraded (37). Inhibition of phosphorylation results in inhibition of IkB degradation. Indeed, sanguinarine inhibits inducible phosphorylation of IkBα and its subsequent degradation.

Interestingly, however, sanguinarine did not block ceramide or TNF-induced NF-κB activation induced by PMA, okadaic acid, and IL-1. Reducing agents like DTT form a complex with the iminium ion of sanguinarine, that it modifies some reactive thiol groups of some protein(s) including some kinases (32). The phosphorylation of IκB has been postulated to be phosphorylated by either the same kinase or by similar kinases (32). The phosphorylation of IkBs results in ubiquinitation at lysines 21 and 22 in IkBα and lysine 22 in IkBβ (32). Polyubiquinitated IkBs are targeted to proteasomes and quickly degraded (37). Inhibition of phosphorylation results in inhibition of IkB degradation. Indeed, sanguinarine inhibits inducible phosphorylation of IkBα and its subsequent degradation.

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