TOXICOLOGICAL SCIENCES **72,** 130–142 (2003)
DOI: 10.1093/toxsci/kfg006

Rapid, Sequential Activation of Mitogen-Activated Protein Kinases and Transcription Factors Precedes Proinflammatory Cytokine mRNA Expression in Spleens of Mice Exposed to the Trichothecene Vomitoxin

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Received July 18, 2002; accepted November 4, 2002

Since proinflammatory cytokine mRNA expression is induced within lymphoid tissue *in vivo* by the trichothecene vomitoxin (VT) in a rapid (1–2 h) and transient (4–8 h) fashion, it was hypothesized that mitogen-activated protein kinases (MAPKs) and transcription factors associated upstream with gene transcription of these cytokines are activated prior to or within these time windows. To test this hypothesis, mice were first treated with a single oral dose of VT and then analyzed for MAPK phosphorylation in the spleen. As little as 1 mg/kg of VT induced JNK 1/2, ERK 1/2, and p38 phosphorylation with maximal effects being observed at 5 to 100 mg/kg of VT. VT transiently induced JNK and p38 phosphorylation over a 60-min time period with peak effects being observed at 15 and 30 min, respectively. In contrast, ERK remained phosphorylated from 15 to 120 min. Next, the binding of activating protein 1 (AP-1), CCAAT enhancer-binding protein (C/EBP), CRE-binding protein (CREB), and nuclear factor-κB (NF-κB) was measured by electrophoretic mobility shift assay (EMSA) using four different consensus transcriptional control motifs at 0, 0.5, 1.5, 4, and 8 h after oral exposure to 25 mg/kg of VT. AP-1 binding activity was differentially elevated from 0.5 h to 8 h, whereas C/EBP binding was elevated only at 0.5 h. CREB binding decreased slightly at 0.5 h but gradually increased, reaching a maximum at 4 h. NF-κB binding was increased only slightly at 4 and 8 h. The specificities of AP-1, C/EBP, CREB, and NF-κB for relevant DNA motifs were verified by competition assays, using an excess of unlabeled consensus and mutant oligonucleotides. Supershift EMSAs and Western blot analysis identified specific VT-inducible DNA binding proteins for AP-1 (cJun, phospho c-jun, JunB, and JunD), C/EBP (C/EBPβ), CREB (CREB-1 and ATF-2), and NF-κB (p50 and cRel). Finally, when the effects of oral VT exposure on proinflammatory gene expression were assessed at 3, 6, and 9 h, splenic TNF-α, IL-1β, and IL-6 mRNA were found to peak at 3 h and were still significantly elevated at 6 h but not at 9 h. Taken together, VT first activated MAPKs *in vivo* and either concurrently (AP-1, C/EBP) or subsequently (AP-1, CREB, NF-κB) modulated binding activities of transcription factors specific for potential regulatory motifs in cytokine promoters. The timing of these events was highly consistent with the kinetics of proinflammatory gene expression in the spleens of mice exposed to VT. This study provides a novel model for studying the interrelationship of MAPK phosphorylation, transcription factor activation, and cytokine gene expression in an intact animal exposed to a toxic compound.

Key Words: **transcription factor; mitogen-activated protein kinases; trichothecene; mycotoxin; vomitoxin; immunotoxicity; TNF-α; IL-1β; IL-6.**

The trichothecenes are a group of 182 structurally related sesquiterpenoid metabolites produced by molds present in food or the environment (Grove *et al*., 1993, 1998, 2000). Notably, there is increasingly widespread contamination of agricultural staples such as wheat, barley, and corn by the fungi *Fusarium graminearum* and *F. culmorum*, apparently due to expanded use of "no-till farming" and changing climate patterns (McMullen *et al*., 1997). The capacity of this fungus to elaborate the trichothecene vomitoxin (VT, deoxynivalenol) in foods at the ppm level is of major human health concern worldwide (Rotter *et al*., 1996).

Leukocytes are primary targets of trichothecenes. Numerous studies of host resistance, mitogen-induced lymphocyte proliferation and humoral immune response have yielded a common theme that trichothecenes can be either immunostimulatory and immunosuppressive, depending on dose, exposure frequency, and timing of functional immune assay (Bondy and Pestka, 2000). Acute high dose trichothecene exposure severely injures actively dividing tissues, including bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa that can result in immunosuppression (Ueno, 1987). Acute low-dose trichothecene exposure aberrantly affects immune function by initiating a rapid and transient upregulation of proinflammatory cytokines (Bondy and Pestka, 2000). These latter effects can be exacerbated by concurrent exposure of experimental animals to a trichothecene and an inflammagenic stimulus such as lipopoly-

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saccharide (Islam *et al*., 2002; Tai *et al*., 1988a,b; Taylor *et al*., 1991; Zhou *et al*., 2000).

The capacity of trichothecenes to upregulate the proinflammatory cytokines TNF-α, IL-1β, and IL-6 appears to drive immunotoxic effects such as shock and IgA nephropathy in mice (Bondy and Pestka 2000). Trichothecene-induced cytokine mRNA expression may be mediated transcriptionally or post-transcriptionally. Relative to the former, altered transcription factor binding activity *in vitro* is of particular significance (Li *et al*., 2000; Ouyang *et al*., 1996; Wong *et al*., 2002). Notably, the TNF-α, IL-1β, and IL-6 promoters all contain binding sites for activating protein (AP-1), CCAAT enhancer-binding protein (C/EBPs), cyclic AMP response element (CRE)-binding protein (CREB, also known as activator transcription factor [ATF]) and nuclear factor-κB (NF-κB) (Godambe, 1994; Haudek *et al*., 1998; Hiscott *et al*., 1993; Husmann *et al*., 1996; Spriggs *et al*., 1992; Tanabe *et al*., 1988).

The underlying molecular toxic event for trichothecenes in eukaryotic cells is believed to involve high affinity binding to peptidyl transferase site of the 60s ribosomal subunit (Middlebrook 1989; Ueno, 1987). Interestingly, translational inhibitors that bind to ribosomes have been observed to rapidly activate mitogen-activated protein kinases (MAPKs) *in vitro* (Iordanov *et al*., 1997), suggesting that these signal transducers may mediate trichothecene toxicity. The three most widely studied MAPK subfamilies are: (1) p44 and p42 MAPKs, also referred to as extracellular signal regulated protein kinase 1 and 2 (ERK 1/2); (2) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2) also known as stress-activated protein kinases (SAPK 1/2); and (3) p38 MAPK (Cobb *et al*., 1999; Widmann *et al*., 1999). MAPKs are important intermediates in signaling pathways that have been implicated in many physiological processes, including cell growth, differentiation, and apoptosis. In support of the contention that these kinases mediate trichothecene immunotoxicity, both our laboratory and others have demonstrated that VT and other trichothecenes activate ERK, JNK and p38 *in vitro* (Anderson, 1999; Shifrin and Moon *et al*., 2002; Yang *et al*., 2000).

A major consequence of MAPK phosphorylation is the activation of transcription factors (Davis, 1995), which serve as immediate or downstream substrates of these kinases. For example, JNK 1/2 phosphorylates c-Jun, which is a component in the AP-1 homodimer or heterodimer (Dong *et al*., 2002). Also, p38 (Bhat *et al*., 2002) and ERK 1/2 (Hungness *et al*., 2002) drive activation of C/EBP. Phosphorylation/activation of CREB/ATF members is mediated by JNK (Dong *et al*., 2002), p38 (Bhat *et al*., 2002) and ERK (Belmonte *et al*., 2001). Finally, all three MAPK signaling pathways have been implicated in nuclear factor κB (NF-κB) activation through phosphorylation of its inhibitor IκBα (Lee *et al*., 1997; Schwenger *et al*., 1998; Zhao and Lee, 1999). It is reasonable to suggest that the capacity of trichothecenes to activate MAPKs may contribute to transcriptional activation of cytokine genes.

Elucidation of how environmental stressors affect global regulation of gene expression and, more specifically, immune function at the kinase and transcription factor levels requires that *in vitro* findings be extended to animal models. Intact animals have been recently used to study the effects of chemical and physiologic stimuli on activation of MAPKs (Hu *et al*., 1997; Suh, 2001; Takagi *et al*., 2000; Yuan *et al*., 1999; Zhong *et al*., 2001a,b) as well as to investigate activation of transcription factors in specific organs (Alam *et al*., 1992; Armstead *et al*., 1999; Ashida and Matsumura, 1998; Blackwell *et al*., 1999; Blazka *et al*., 1996; Zhong *et al*., 2001a,b). These studies suggest that it might be feasible to integrate both strategies to study the molecular effects of trichothecenes *in vivo*.

Cytokine mRNAs and proteins are typically induced by VT *in vivo* in rapid (1–2 h) and transient fashions (4–8 h) (Azcona *et al*., 1995; Zhou *et al*., 1998; Zhou and Pestka, 1997;). Here, we hypothesized that MAPKs and transcription factors are activated prior to or concurrently with proinflammatory cytokine gene upregulation *in vivo*. To test this hypothesis, mice were treated orally with VT and their spleens analyzed temporally for MAPK phosphorylation as well as for activation of splenic nuclear proteins binding to four different consensus transcriptional control motifs associated with cytokine promoters. Response elements associated with AP-1, C/EBP, CREB, and NF-κB were selected based on the presence of these DNA sequences in the TNF-α, IL-1β and/or IL-6 promoters as well as on the recognized capacities of these transcription factors to mediate proinflammatory cytokine gene transactivation *in vitro*. The results indicated that VT induced the rapid and transient phosphorylation of ERK, p38 and JNK *in vivo*. These effects were concurrent with or followed by time-dependent increases and decreases in transcription factor binding corresponding to nuclear translocation. Both timing and differential activation of MAPKs and transcription factors by VT were consistent with the profile, magnitude, and duration of proinflammatory cytokine expression described previously and confirmed in this study.

MATERIALS AND METHODS

General experimental design. All animal studies followed NIH guidelines and were approved by the Michigan State University Committee on Animal Care. Eight- to ten-week-old male B6C3F1 mice (Charles River, Portage, MI) were used in the study. Prior to the experiment, animals were held for a 1-week acclimation period in a room with a 12-h light/dark cycle, 70% humidity, and a temperature of 22–23°C.

For MAPK studies, mice were administered VT (1 to 100 mg/kg) by a single oral gavage in 0.25 ml of endotoxin-free water. These doses were selected to bracket VT doses of 5 to 25 mg/kg, which are sufficient to induce strong proinflammatory cytokine responses in this mouse model (Azcona *et al*., 1995; Zhou *et al*., 1997, 1998). Control mice were given 0.25 ml of endotoxin-free water. Mice were killed after 15, 30, 60, and 120 min by cervical dislocation under methoxyflurane (Schering-Plough Animal Health, Union, NJ). Spleens were removed and whole cell lysates analyzed for MAPK phosphorylation.

For transcription-factor studies, mice were gavaged orally with 25 mg/kg of VT. They were then killed at 0.5, 1.5, 4, and 8 h (kinetic studies) or at 0.5, 1.5,

or 4 h post-treatment (competition, supershift, and Western blot analyses) and spleens were excised and pooled (2–3 per group) for preparation of nuclear protein extracts to conduct EMSA.

For cytokine mRNA studies, mice were gavaged orally with 12.5 mg/kg of VT. Spleens were removed at 0, 3, 6, and 9 h and analyzed for proinflammatory cytokine mRNA.

Detection of MAPK phosphorylation. Phosphorylation of MAPKs was assayed by Western blot, using rabbit polyclonal antibodies specific for phospho-JNK 1/2, phospho-ERK 1/2, and phospho-p38 MAPK (Cell Signaling, Beverly, MA) as described in manufacturer's instruction. Spleen cells were dispersed in SDS lysis buffer (1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris pH [7.4]), transferred to a microcentrifuge tube, boiled for 5 min, and then sonicated briefly. The lysate was centrifuged at 15,000 × *g* for 15 min at 4°C to pellet insoluble material. Protein concentration of the resultant supernatant was determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Inc., Melville, NY). Total cellular proteins were resolved by 8% (w/v) SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, Arlington Heights, IL). After blocking with 5% (w/v) nonfat dry milk, the immobilized proteins were incubated with phospho-specific antibodies followed by horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Amersham). Bound peroxidase was determined using an ECL chemiluminescence detection kit (Amersham). To assess loading, membranes were stripped and reprobed with specific antibodies that recognize both phosphorylated and unphosphorylated forms (Cell Signaling) of each MAPK.

Preparation of nuclear extracts. Spleen cells were dissociated and passed through a 100 mesh stainless steel screen. Cells were suspended in Dulbecco's phosphate buffered saline (PBS, Sigma). Erythocytes were lysed for 2 min at 25°C in 0.01 M $KHCO_3$ containing 0.14 M NH_4Cl. Nuclear extracts were prepared using the method of Olnes and Kurl (1994) with modifications to prevent protein modification or degradation. Briefly, cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM Mg Cl_2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol) with phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin A, 2 mg/ml leupeptin, 2 mg/ml aprotinin), and 0.6% (w/v) Nonidet P-40. Nuclei were pelleted by centrifugation, suspended in hypertonic buffer containing 20 mM HEPES pH 7.9, 0.4 M KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 10% (w/v) glycerol, and the phosphatase and protease inhibitors indicated above. After 30 min at 4°C, soluble proteins released were collected by centrifugation at 15,000 × *g* for 10 min. The supernatant was dialyzed for 2 h at 4°C against 1000 volumes of dialysis buffer (20 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% [w/v] glycerol supplemented with leuprotinin, aprotinin, and sodium fluoride). Resultant extracts were analyzed for protein, aliquoted, and then stored at −80°C until analysis.

Electrophoretic mobility shift assay (EMSA). EMSA was used to characterize the binding activity of AP-1, C/EBP, CREB, and NF-κB transcription factors in nuclear extracts (Chodosh, 1993). Double stranded AP-1, C/EBP, CREB, and NF-κB consensus probes (Santa Cruz Biotech, Santa Cruz, CA) (Table 1) were radiolabeled with [γ-^{32}P-ATP] using Ready to Go™ Polynucleotide Kinase Kit (Pharmacia Biotech, Inc., Piscataway, NJ). Nuclear extracts containing 5 to10 μg protein were added to DNA-binding reaction buffer consisting of 20 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA, 0.5 mM DTT, 2 μg poly dIdC in a total volume of 20 μl. These were preincubated on ice for 15 min to block nonspecific binding. Following the addition of 1 μl ^{32}P-labeled probe containing 30,000 cpm, the incubation was continued for 30 min at room temperature to promote the formation of nucleoprotein complexes. Resultant nucleoprotein complexes were analyzed by loading samples on a 4% (w/v) native polyacrylamide gel in 0.5 × TBE buffer, dried, and visualized by autoradiography. In each set of experiments, a self-competition was performed by adding excess of the same unlabeled oligonucleotide probe or a corresponding mutant oligonucleotide probe (Table 1) to the binding reaction at the preincubation step.

EMSA supershift experiments were conducted using specific antibodies to AP-1(c-Jun, JunB, JunD, phosphorylated c-Jun, c-Fos, Fos B, Fra-1, and Fra-2), C/EBP (C/EBPα, C/EBPβ, and C/EBPδ), CREB (CREB-1, CREB-2, ATF-2 and ATF-3), and NF-κB (p65, p50, c-Rel, and Rel-B) or, as a control, 2 mg rabbit IgG (Santa Cruz Biotech). Reactions were identical to those described above, except 1–2 μg of appropriate antibody was added to the binding reactions after addition of the labeled probe and the reactions were incubated overnight at 4°C prior to electrophoresis.

TABLE 1
Sequences of Consensus Oligonucleotides Used in Electrophoretic Mobility Shift Assay

Consensus	Substitutions in mutant
AP-1 5′-CGGTGA**TGACTCA**GCCGGAA-3′	CA → TG
C/EBP 5′-TGCAGA**TTGCGGAA**TCTGCA-3′	TTGCGGAA → GACTAGTC
CREB 5′-AGAGATTGCC**TGACGTCA**GAGAGCTAG-3′	AC → TG
NF-κB 5′-AGTTGA**GGGGACTTT**CCCAGGC-3′	G → C

Western blot analysis of nuclear transcription factors. Electrophoresis of nuclear proteins was performed with 10% (w/v) polyacrylamide gel in the presence of 0.1% SDS in the discontinuous buffer system of Laemmli (1970). The proteins were transferred to a PVDF membrane (Amersham, Piscataway, NJ). Membranes were incubated with polyclonal antibodies for C/EBP, AP-1, CREB, and NF-κB family members, washed and then incubated with horseradish-peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham). Enzyme activity in bands was visualized using chemiluminescence as described for MAPKs.

RNA isolation and quantitation of cytokine mRNA. Total RNA from spleen was isolated using an RNAqueous Kit (Ambion Inc., Austin) according to the manufacturer's protocol. The resulting RNA was dissolved in 50 μl of elution buffer and stored at −80°C. All PCR reactions for IL-6 and TNF-α cytokine mRNA quantification were performed on an ABI PRISM 7700 Sequence Detector System using Taqman One-Step RT-PCR Master Mix Reagents Kit according to the manufacturer's protocol (Applied Biosystems). *C*t values for Il-6, TNF-α and 18S rRNA were adjusted using the standard curves of known amounts of total RNA (ranging 1.37 to 1000 ng/) and normalized by dividing either IL-6 or TNF-α adjusted amounts by the 18S adjusted amount. IL-1β cytokine mRNA was quantitated by Quantikine colorometric ELISA method (R&D systems, Inc., Minneapolis) according to the manufacturer's protocol.

Statistics. The data were analyzed by Dunnett's and Student-Neuman-Keuls tests using Sigma-Stat Statistical Analysis System (Jandel Scientific, San Rafael, CA). A p value of <0.05 was considered statistically significant.

RESULTS

Acute oral VT exposure induces MAPK phosphorylation in spleen. Our laboratory has previously determined that VT rapidly induces activation of MAPKs in macrophages *in vitro* (Moon *et al*., 2002). To assess the effects of VT on MAPK activation *in vivo*, mice were gavaged orally with 0, 1, 5, 25, and 100 mg/kg of VT, and, after 15 min, their spleen cell lysates evaluated for MAPK phosphorylation by Western blot analysis. As little as 1 mg/kg of VT was found to be effective at inducing JNK 1/2, ERK 1/2 and p38 phosphorylation (Fig. 1) Maximal phosphorylation of all three MAPK families occurred at 5 mg/kg VT; this level of phosphorylation was maintained at higher concentrations. When Western blot assays were conducted with antibodies reactive to both phosphory-

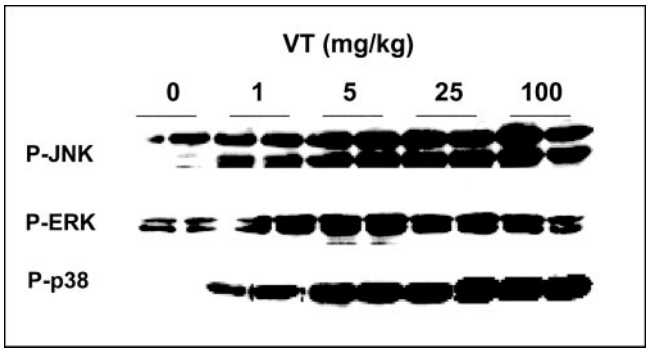


FIG. 1. VT induces MAPK phosphorylation in the murine spleen. Mice were treated with vehicle or VT by oral gavage (2 mice/dose). After 15 min, mice were killed, spleens removed, and tissue extract prepared. Extracts were analyzed for MAPK activation by Western analysis using antibodies to phosphorylated JNK, ERK, and p38.

lated and non-phosphorylated MAPKs, VT was found to have no effect (data not shown).

The kinetics of MAPK phosphorylation in spleen cells was evaluated over a 2-h period following oral gavage with 25 mg/kg VT. JNK 1/2 and p38 activation were transient, with maximal phosphorylation being detectable at 15 to 30 min (Fig. 2). A MAPK phosphorylation response at an earlier time point, 10 min, was weak or nonexistent among mice tested (data not shown). Greatly reduced phosphorylation of JNK 1/2 and p38 were observed at 60 and 120 min. ERK 1/2 activation was more prolonged, with maximal phosphorylation being detectable in spleen and thymus from 15 to 60 min with only a slight reduction at 120 min. Basal MAPK levels were unaffected by VT treatment (data not shown). Thus, as has been observed *in vitro*, VT at doses as low as 1 mg/kg rapidly induced phosphorylation of MAPKs in murine spleen *in vivo*.

VT activates AP-1 binding and nuclear translocation by VT. . VT has been previously shown to activate AP-1 binding and nuclear translocation in macrophages (Wong *et al*., 2002) and

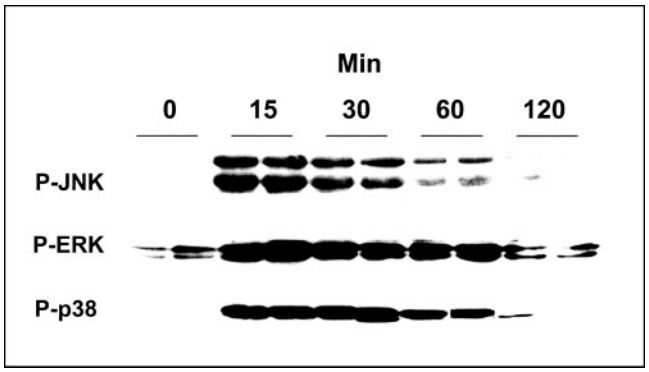


FIG. 2. Kinetics of MAPK activation by VT *in vivo*. Mice were treated orally with 25 mg/kg VT. After designated time interval, spleen was removed, homogenized, and subjected to Western analysis with JNK, ERK, and p38-specific antibodies.

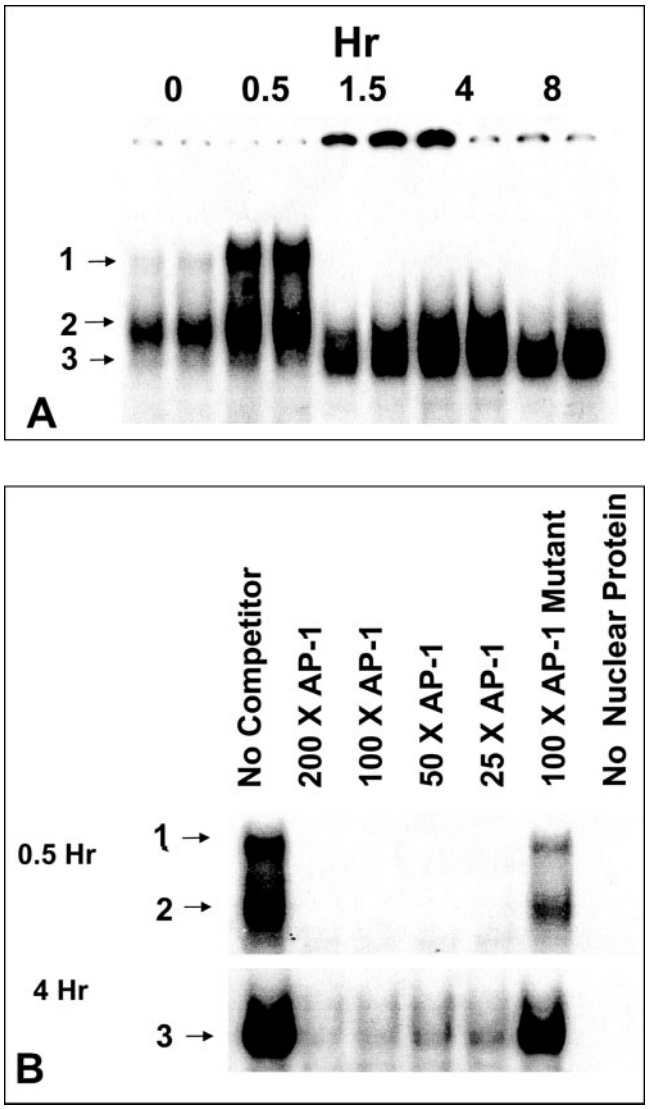


FIG. 3. Effect of VT on splenic AP-1 binding activity. (A) Kinetics of AP-1 binding response in mouse spleen after oral gavage with VT. Mice were gavaged orally with VT (25 mg/kg bw) or vehicle (endotoxin-free water). Cells were isolated from the spleen at 0.5, 1.5, 4, and 8 h and nuclear extracts prepared. Binding activities of nuclear extracts (5 μg) were analyzed by EMSA using a ^{32}P-labeled, double-stranded AP-1 consensus probe. (B) Specificity of AP-1 binding in 0.5 ml 4-h extracts. Nuclear extracts (5 μg) were subjected to EMSA using the ^{32}P-labeled AP-1 consensus oligonucleotide (30,000 cpm) with the addition of excess unlabeled consensus or mutant probe, prior to the adding of ^{32}P-labeled oligonucleotide. No nuclear protein, assays were run without added nuclear protein.

T cells (Li *et al*., 2000). Here, the effect of a single 25 mg/kg oral VT exposure in the mouse on splenic protein binding to the consensus sequence was assessed over an 8 h time period. EMSA revealed three predominant bands in the various experimental groups (Fig. 3A). Nuclear extracts from naive mice showed only a single AP-1 band (Band 2), whereas extracts from VT-treated mice exhibited Band 2 and a slower migrating band (Band 1) at 0.5 h. Band 1 was not evident in VT-treated

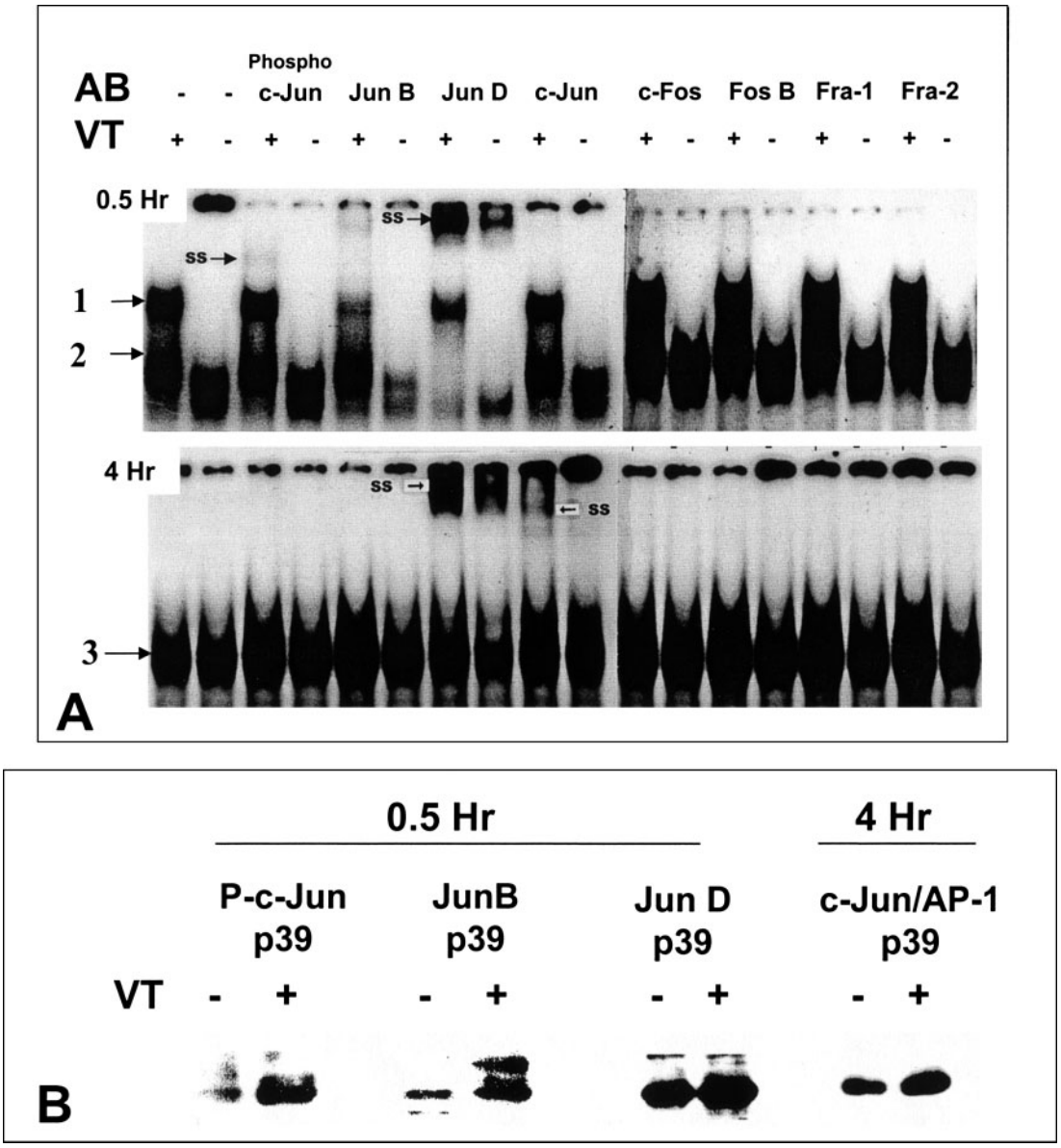


FIG. 4. Characterization of the VT-inducible AP-1 subunits. (A) Supershift EMSA was performed using nuclear extracts from 0.5 and 4-h treatment as described in the Figure 3 legend, except that 1–2 μg specific antibodies were added to the reaction mixture after adding ^{32}P-labled probe (50,000 cpm) and incubated overnight at 4°C prior to electrophoresis. Antibodies were directed against phosphorylated (phospho) c-Jun, cJun, JunB, JunD, FosB, cFos, Fra-1, and Fra-2. SS indicates supershift band. (B) Western blot analysis was conducted after SDS–PAGE of nuclear protein from 0.5 and 4.0-h treatments. Primary rabbit antibodies were directed against the subunits of AP-1 conjugates and secondary antibody was a horseradish peroxidase donkey anti- rabbit IgG antibody conjugates. Immune complex bands were visualized by chemiluminescence.

mice at 1.5 h or thereafter. A third band (Band 3) appeared after 1.5 h, peaked at 4 h and declined slightly at 8 h. Changes in band appearance were not observed in mice given vehicle and analyzed over the 8 h time period (data not shown). The specificity of all three bands was verified by complete competition with $\geq$25 $\times$ excess unlabeled oligonucleotide probe but not unlabeled mutant probe (Fig. 3B).

Supershift EMSAs performed on 0.5-h nuclear extracts indicated that Bands 1 and 2 were depleted and a shifted band was detectable in mixtures incubated with JunD-specific antibody (Fig. 4A). A faint supershift band was also detectable after incubation with antibody specific for phosphorylated c-Jun. Band 1 was markedly depleted upon incubation with JunB antibody. This is likely the result of the antibody binding to the transcription factor binding site and inhibiting complex formation. After 4 h, supershift bands were detected for c-Jun and JunD. Incubation with normal IgG had no effect (data not shown). Western blot analysis verified that all four of the aforementioned Jun family members were present at higher concentrations in nuclear extracts from VT-treated mice than

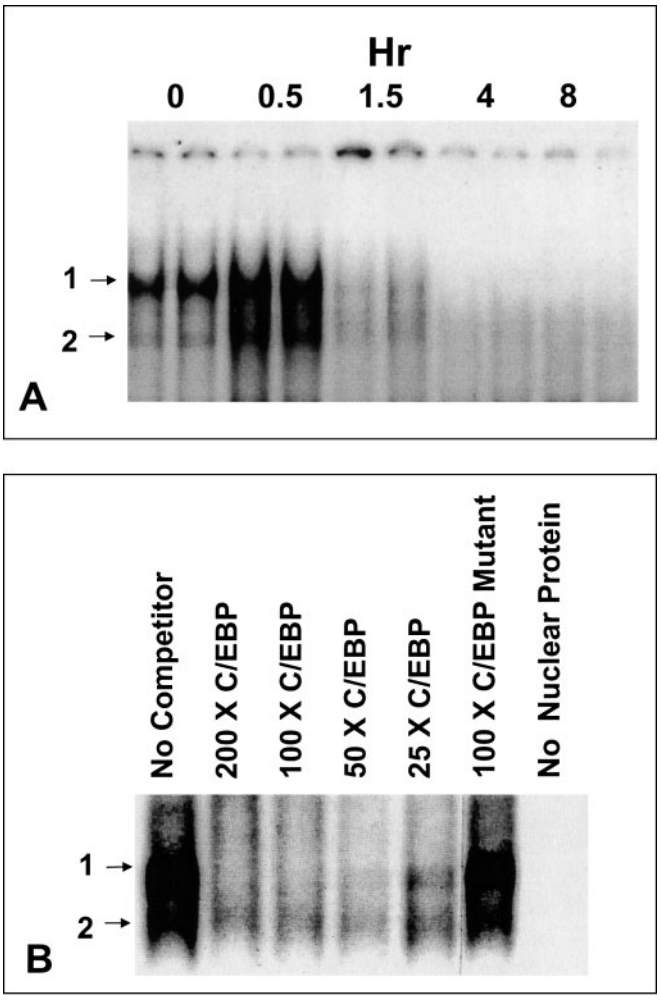


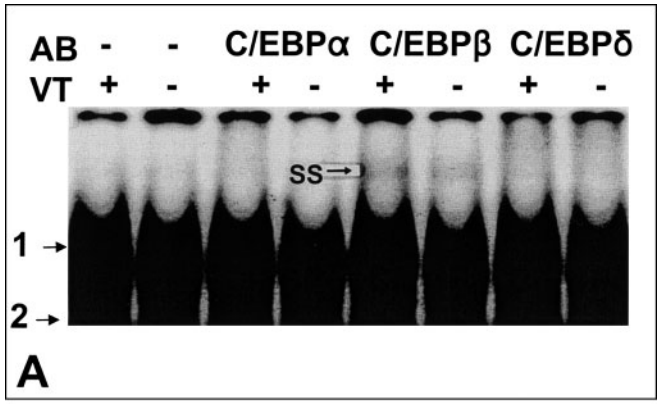
FIG. 5. Effect of VT on splenic C/EBP binding activity. (A) Kinetics of C/EBP binding response in mouse spleen after ip oral gavage with 25 mg/kg bw VT. EMSA was performed as described in the Figure 3A legend, except a C/EBP consensus probe was used. (B) Specificity of C/EBP in nuclear extracts from 0.5 h VT-treatment group. The competitive EMSA was assessed as described in Fig. 3B.

from vehicle-treated mice, thus confirming supershift results (Fig. 4B). In contrast to Jun, Fos family members were not identified in the AP-1 complexes by supershift (Fig. 4A). Furthermore, Western analyses revealed that VT had no effect on nuclear translocation of c-Fos, FosB, Fra-1, or Fra-2 (data not shown). Thus, VT caused a marked increase in AP-1 binding activity that exclusively involved Jun protein dimers, with transient Band 2 likely being a JunB homodimer, Band 1 possibly being composed of one or more dimers of JunD, JunB, and phospho c-Jun; and Band 3 containing c-Jun and JunD.

VT activates C/EBP binding and nuclear translocation. Incubation of splenic nuclear extracts with the C/EBP consensus oligonucleotide probe resulted in two major bands (Fig. 5A). A slow migrating band (Band 1) and a weak faster migrating band (Band 2) were observed in naive mice. Marked increases in Band 1 and Band 2 were observed 0.5 h after VT-treatment. Bands 1 and 2 were undetectable from 1.5 to 8 h. Vehicle-treated mice exhibited no effect over the time course (data not shown). In VT-treated animals, specificity of Bands 1 and 2 were verified by complete competition with $\geq 50 \times$ excess unlabeled oligonucleotide probe but not mutant unlabeled probe (Fig. 5B).

EMSAs using nuclear extracts with radiolabeled C/EBP probe and specific antibodies revealed a faint supershift band corresponding to C/EBPβ but not C/EBPα or C/EBPδ (Fig. 6A). Incubation with normal IgG also had no effect (data not shown). Western blot analysis confirmed a markedly higher concentration of C/EBPβ in nuclear extracts from LPS-treated mice as compared to vehicle-treated mice (Fig. 6B), but this was not observed for C/EBPα or C/EBPδ (data not shown). The results suggest that VT exposure *in vivo* elevated C/EBP binding activity at 0.5 h, predominantly through increased nuclear C/EBPβ, but subsequently evoked a nearly complete loss of detectable binding activity thereafter.

VT activates CREB binding and nuclear translocation. EMSA with a CREB consensus sequence yielded an intense band (Band 3) and suggested faint slow (Band 1) and fast



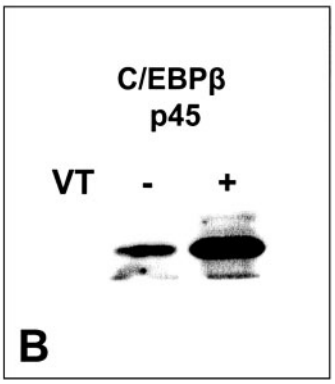


FIG. 6. Characterization of the VT-inducible C/EBP subunits. (A) Supershift EMSA using nuclear extracts from 0.5-h treatment as described in the Figure 4A legend. Antibodies were directed against C/EBPα, C/EBPβ, and C/EBPδ. (B) Western blot analysis after SDS–PAGE of nuclear proteins from 0.5-h treatment was conducted as described in Figure 4B.

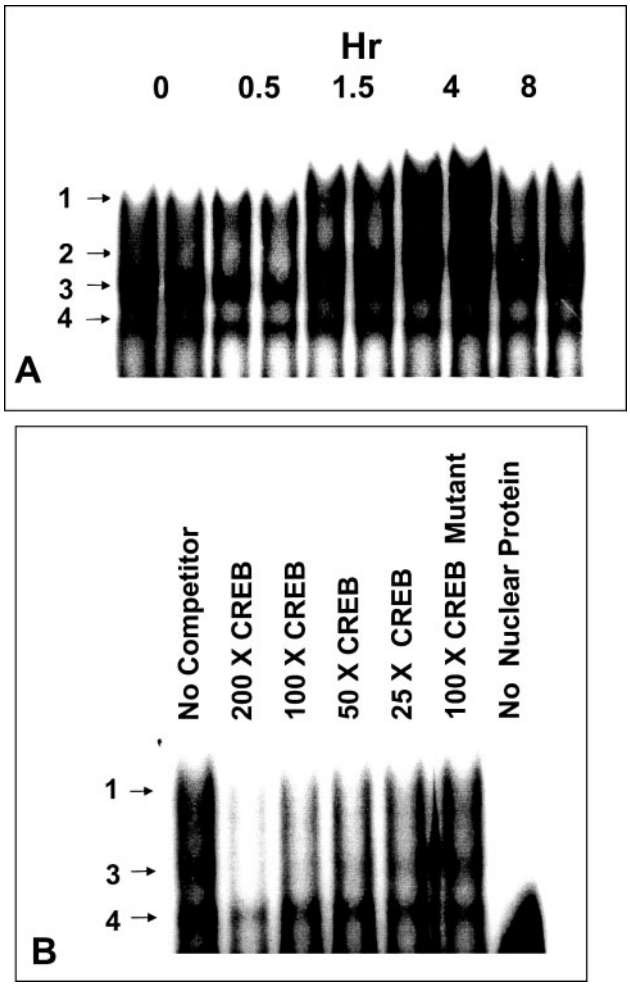


FIG. 7. Effect of VT on splenic CREB binding activity. (A) Kinetics of CREB binding response in mouse spleen after oral gavage with VT. EMSA was performed as described in the Figure 3A legend. Specificity of CREB in nuclear extracts from a 0.5-h treatment. The competitive EMSA was as described in Figure 3C.

(Band 3) migrating bands in naive mice. These were detectable and unchanged in vehicle control groups over 8 h (data not shown). Band 3 intensity decreased 0.5 h after VT treatment, but returned to control values after 4 and 8 h. At 4 and 8 h, intensity Band 1 increased markedly, and a third band (Band 2) appeared. Competition with $\geq 50 \times$ excess unlabeled oligonucleotide probe but not mutant probe completely inhibited Bands 1, 3, and 4 in a 0.5 h extract, thus suggesting these to be specific for the CREB response element (Fig. 7B).

Incubations of nuclear extracts with antibodies to the CREB family members CREB-1 and ATF-2 were found to induce faint shifted bands and, furthermore, slight depletion of Band 1 by anti-ATF-2 (Fig. 8A). Normal IgG had no effect (data not shown). Elevated nuclear CREB-1 and ATF-2 were confirmed in VT-treated mice as compared with vehicle-treated mice (Fig. 8B). Nuclear translocation of CREB-2 and ATF-3 was not observed following VT treatment (data not shown). These data suggest that the toxin evoked a marked loss of detectable constitutive CREB binding activity at 0.5 h but increased activities at 1.5 and 4 h, possibly through increased nuclear CREB-1 and ATF-2.

VT activates NF-κB binding and nuclear translocation. Using a κB consensus sequence, EMSA resulted in two major bands in the experimental groups (Fig. 9A). Nuclear extracts from control mice exhibited a faint fast migrating band (Band 2) and a stronger, slow migrating band (Band 1), both of which remained constant over the 8 h time period (data not shown). In VT-treated mice, Bands 1 and 2 were largely unaffected at 0.5 and 1.5 h but were markedly elevated at 4 and 8 h. Specificity of Band 1 was verified by nearly complete competition with $\geq 100 \times$ excess unlabeled oligonucleotide probe but not mutant unlabeled probe (Fig. 9B).

Anti- p50 depleted Band 1 in supershift NF-κB EMSAs from nuclear extracts from both VT- and vehicle-treated mice and generated one shifted band (Fig. 10A). Although incubation with anti-c-Rel caused no apparent depletion in Bands 1 or 2, a faint-shifted band was detectable in spleens by VT-treated animals. Antibodies to p65 or Rel B had no effect on EMSA results (data not shown). Western blot analysis indicated slight increases in NFκB p50 and c-Rel in nuclear extracts from

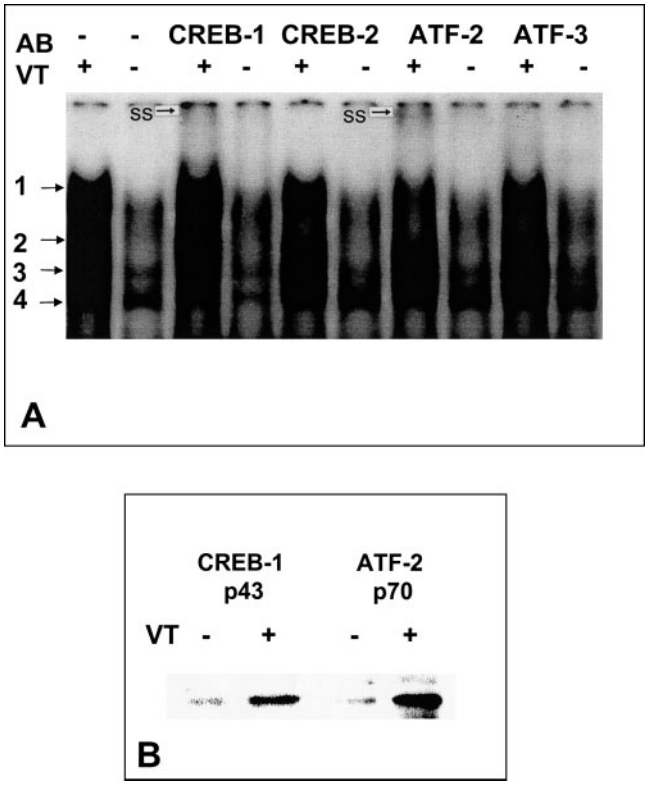


FIG. 8. Characterization of VT-inducible CREB subunits. (A) Supershift EMSA using nuclear extracts from a 4-h treatment, as described in Figure 4A. Specific antibodies were: CREB-1, CREB-2, ATF-2, and ATF-3. (B) Western blot analysis after SDS–PAGE of nuclear proteins from 4 h was conducted as described in Figure 4B.

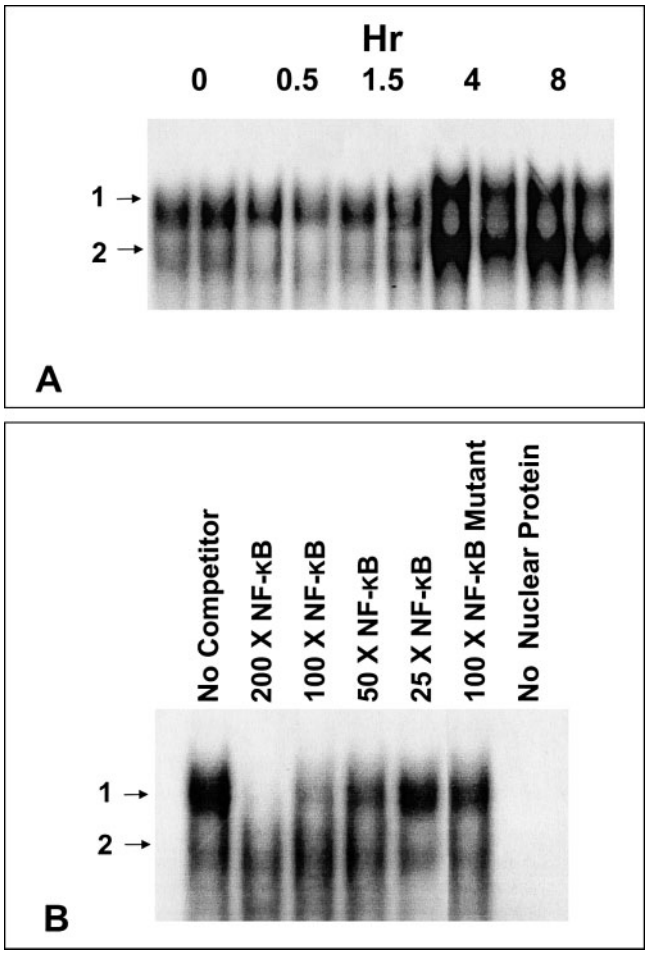


FIG. 9. Effect of VT on splenic NF-κB binding activity. (A) Kinetics of NF-κB binding response in mouse spleen after oral gavage with VT. The EMSA was performed as described in Figure 3A, except that NF-κB consensus probe was used. (B) Specificity of NF-κB in nuclear extracts from a 1.5-h treatment. Competitive EMSA was as described in Figure 3B.

VT-treatment compared to vehicle-treatment groups (Fig. 10B). Taken together, the results suggest that VT treatment *in vivo* caused a late transient increase in NF-κB binding activity that correlated with elevated nuclear p50 and cRel. Band 1 likely consisted of a p50 homodimer and/or heterodimers of p50 complexed with cRel.

VT transiently induces proinflammatory cytokine mRNA expression. In previous studies, we have determined that VT induces proinflammatory gene expression in spleen in rapid and transient fashion (Azcona *et al*., 1995; Zhou *et al*., 1997). These effects were verified in the context of the current model using recently developed quantitative methods for mRNA. Specifically, mice were treated with a single dose of VT (12.5 mg/kg) and proinflammatory cytokine mRNAs measured at 3, 6, and 9 h. TNF-α mRNA expression was 3- and 2-times the control value at 3 and 6 h, respectively (Fig. 11). Similarly, splenic IL-1β mRNA expression was 1.8 and 1.4-times the control value at 3 and 6 h, respectively. VT's affects were greatest on IL-6 mRNA which was 17- and 2-times control value at 3 and 6 h, respectively. The mRNA levels for all three proinflammatory cytokines returned to control levels at 9 h. These results were consistent with previous findings relative to magnitude of expression and kinetics using semiquantitative reverse transcriptase-PCR in conjunction with Southern hybridization (Zhou *et al*., 1997).

DISCUSSION

This study is novel because it is the first attempt, to our knowledge, at temporally relating MAPK phosphorylation, transcription factor activation, and cytokine gene expression in intact animals exposed to a toxin. Previous studies reported by our lab and confirmed here indicate that VT drives proinflammatory cytokine gene responses in the mouse spleen within 1 to 2 h and that the response is transient, ceasing within 4 to 8 h (Table 2) (Azcona *et al*., 1995; Zhou *et al*., 1997). The results presented herein suggest that VT induced MAPK phosphorylation after only 15 min (Table 2). Furthermore, induc-

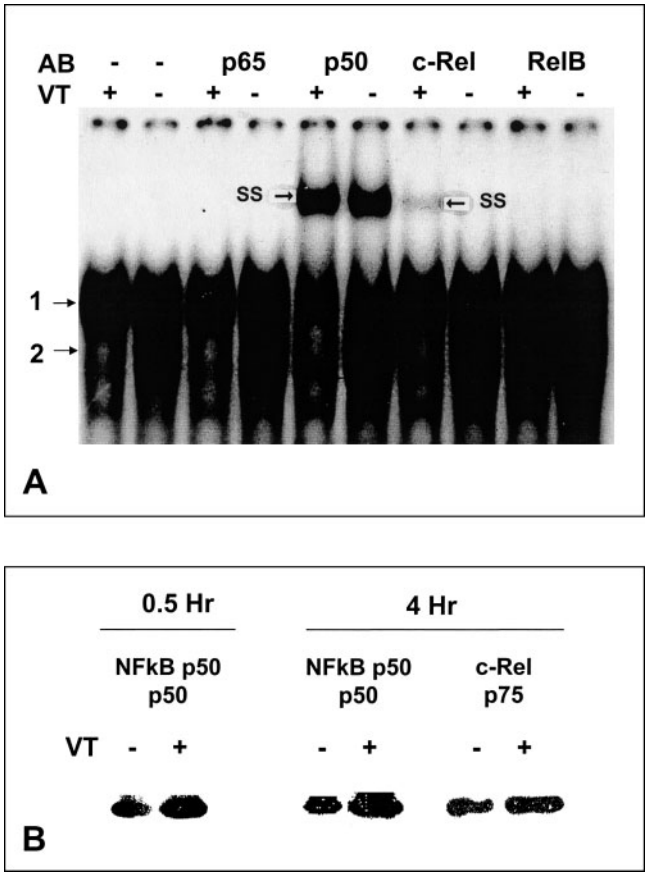


FIG. 10. Characterization of VT-inducible NF-κB subunits. (A) Supershift EMSA used nuclear extracts from a 1.5-h treatment, as described in Figure 4A. Specific antibodies were directed toward p65, p50, c-Rel, and RelB. (B) Western blot analysis after SDS–PAGE of nuclear proteins from 0.5- and 4-h treatments were conducted as described in Figure 4B.

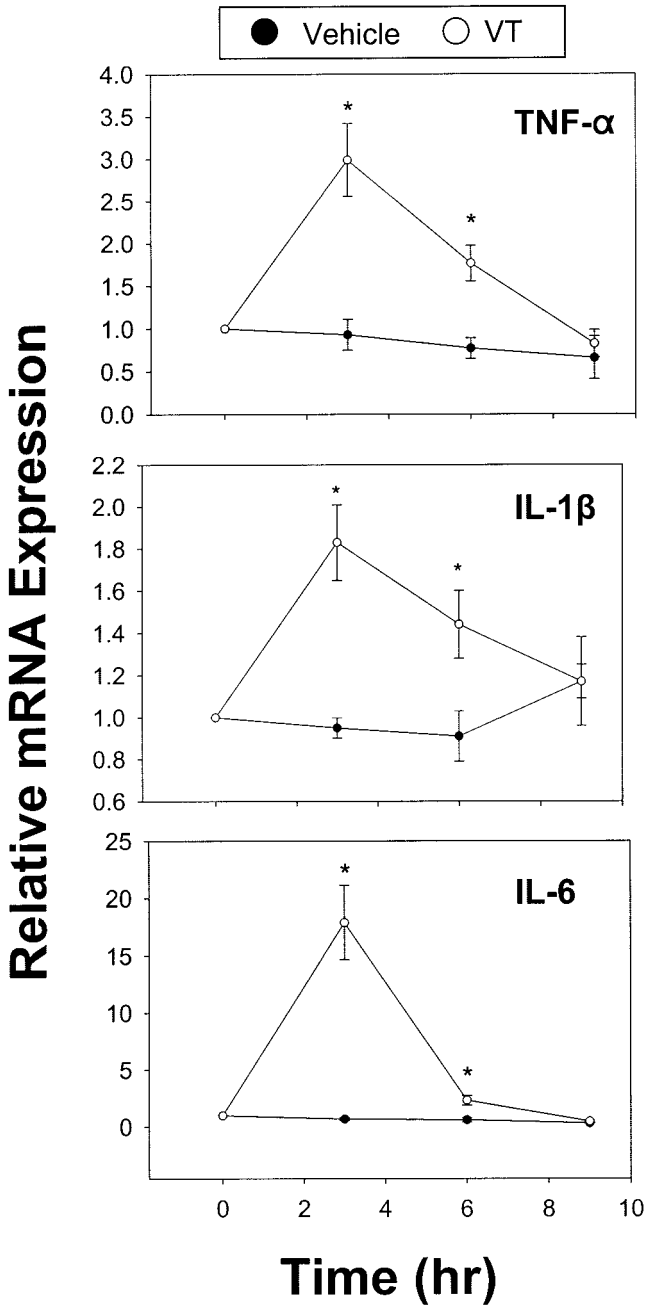


FIG. 11. VT induces TNF-α, IL-β, and IL-6 splenic mRNA. Mice were orally gavaged with VT (12.5 mg/kg bw) or vehicle. Total splenic RNA was isolated at time intervals and quantitatively analyzed for proinflammatory cytokine mRNAs. Data are mean ± SEM ($n = 6$) of the increase relative to the zero time control. *Significantly different from zero time and vehicle control.

tion of at least one AP-1 complex (Band 1) and the C/EBP complex occurred concurrently with or shortly after phosphorylation of the MAPKs, whereas activation of another AP-1 complex, as well as CREB and NF-κB complexes, was later and more prolonged. Overall, these data are consistent with a model whereby trichothecenes first induce MAPK phosphorylation, which, in turn, activate two transcription factors (AP-1, C/EBP) that contribute to elevated proinflammatory cytokine gene expression (Table 2, Fig. 12). The subsequent and more prolonged activation of AP-1, CREB, and NF-κB might be responsible for maintaining, extending, and/or downregulating TNF-α, IL-1β, and IL-6 mRNA responses. These later effects might be driven directly by VT or indirectly by cytokines generated in the initial response via receptor-mediated pathways.

The results suggest that exposure to 1 mg/kg VT was sufficient to activate all three MAPK families. This would be equivalent, in the mouse, to consuming a 5 g meal containing 5 ppm of the toxin. In previous work, we have observed national brand granola and corn meal samples to contain more than 15 ppm of VT (Abouzied *et al.*, 1991). Relatedly, Li *et al.* (1999) has reported that in a food poisoning outbreak in China, VT concentrations of up to 51 ppm were detectable in corn consumed by affected persons. Thus, the doses employed in the present study are relevant to human exposure. From the perspective of toxin disposition and clearance, the rapid and transient MAPK response observed here was not surprising in light of an earlier study we conducted in B6C3F1 mice (Azcona *et al.*, 1995). In that paper, it was observed that, following oral gavage with ^{3}H-VT, maximal levels of the label were found in spleen, plasma, and five other tissues within 30 min, the earliest time point tested. At the 25-mg/kg body weight (bw) dose, VT clearance followed two-compartment kinetics with an initial rapid clearance ($t_{1/2} = 0.56$ h) and in slower terminal elimination ($t_{1/2} = 88.9$ h) within 24 h, 88% of the toxin was removed from plasma at this dose. These results

TABLE 2

Temporal Relationships for VT-induced MAPK Phosphorylation, Transcription Factor Activation, and Proinflammatory Cytokine Expression in the Mouse Spleen

Event	Earliest detectable effect (h)	Peak effect (h)	Latest detectable effect (h)
MAPK phosphorylation[a]			
JNK 1/2	0.25	0.25	1.0
ERK 1/2	0.25	0.25	≥2.0
p38	0.25	0.25–0.50	1.0
Transcription factor activation[a]			
AP-1 (BAND 1)	0.5	0.5	0.5
AP-1 (BAND 3)	1.5	4.0	≥8.0
C/EBP	0.5	0.5	0.5
CREB (BAND 2)	1.5	4.0	4.0
CREB (BAND 3)	1.5	4.0	4.0
NF-κB	4.0	4.0–8.0	≥8.0
Proinflammatory cytokine mRNA expression[a,b]			
TNF-α	1.0–3.0	2.0–4.0	6.0–8.0
IL-1β	1.0–3.0	2.0–3.0	4.0–6.0
IL-6	2.0–3.0	2.0–4.0	6.0–8.0

[a]Data are from this study.

[b]Data are from Azcona-Olivera *et al.* (1995) and Zhou *et al.* (1997).

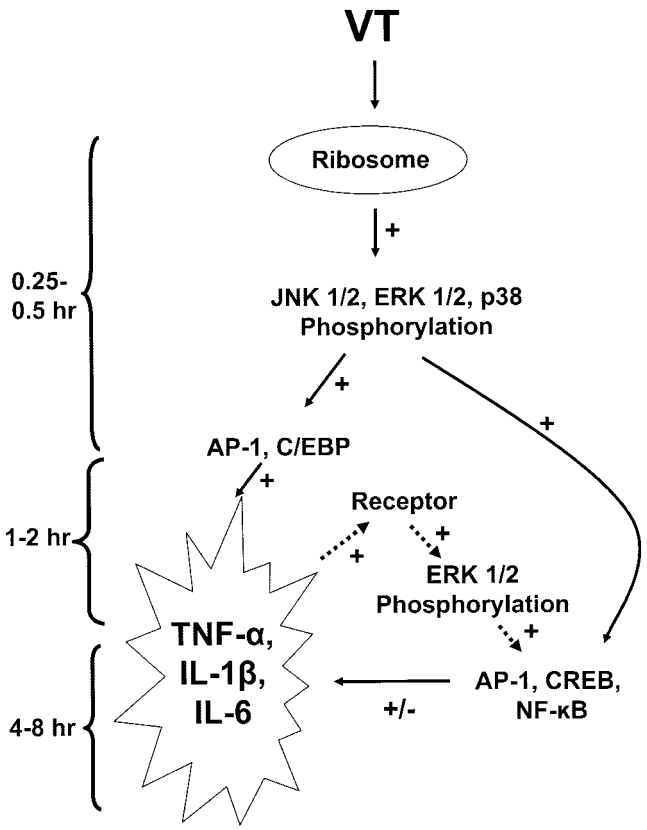


FIG. 12. Hypothetical pathways linking VT-induced MAPK and transcription factor activation to induction of proinflammatory cytokine gene expression. Solid line indicates possible intracellular signaling events leading to cytokine upregulation that are suggested by this study. Dashed line indicates potential external signaling pathway mediated by autocrine or paracrine stimulation by secreted cytokines.

suggest that the transient MAPK and transcription factor responses observed herein may be readily explained by rapid disposition and clearance of VT, the underlying stimulus.

Although the exact mechanisms by which VT induces MAPK phosphorylation remain unresolved, trichothecenes (Shifrin and Anderson, 1999; Yang *et al*., 2000) and other translational inhibitors (Iordanov *et al*., 1997), which bind to eukaryotic ribosomes known to activate MAPKs. Iordanov *et al*. (1997) found that anisomycin and antibiotics that bind to 28S rRNA are strong activators of JNK. Notably, two ribotoxic enzymes, ricin A chain, and alpha-sarcin, both of which catalyze sequence-specific RNA damage in the 28S rRNA, are strong agonists of JNK1 and of its activator, SEK1/MKK4. Anisomycin and the ribotoxic enzymes apparently mediate signal transduction from altered 28S rRNA to JNK in active ribosomes but not in inactive ribosomes. These investigators described the capacity of the ribosomes to sense cellular stress as a "ribotoxic stress response." The possibility exists that VT's capacity to activate MAPKs *in vitro* and *in vivo* is also reflective of a ribotoxic stress response, and that it drives subsequent events related to transcription factor activation and ultimately, cytokine expression. The molecular transducer linking ribosomal binding of trichothecene and the almost immediate activation of MAPKs is not known and represents a fertile area for future investigation.

JNK 1/2 and p38 are generally believed to be stress responsive MAPKs and to be susceptible to chemicals, while ERK is thought activated by receptor-mediated mechanisms. In contrast, our results suggest that ERK can be rapidly activated by VT and confirm findings that have been made *in vitro* with VT (Moon *et al*., 2002) and other trichothecene mycotoxins (Yang *et al*., 2000). It is possible that the prolonged ERK response, as compared to JNK and p38, may be receptor-driven via the cytokines that are initially induced by VT (Fig. 12).

AP-1 regulates many genes associated with proinflammatory cytokine production, monocytic differentiation, B- and T-cell activation, Th1/Th2 differentiation of immunoglobulin production, and apoptosis (Pennypacker, 1998). AP-1 binds to the palindromic TRE sequence TGA(C/G)TCA (Liebermann *et al*., 1998). Members of the AP-1 family include four Fos proteins (c-Fos, Fos B, Fra-1, and Fra-2) and three Jun proteins (c-Jun, JunB, and JunD) (Pennypacker, 1998). These can interact as either a Jun homodimeric complex or as a Fos/Jun heterodimeric complex. The seven subunits are subject to regulation via phosphorylation and by chemical oxidation of specific cysteine residues mapping within the DNA binding domains of Fos and Jun. Our results suggest that VT exclusively affected Jun homodimers including a JunB homodimer as well as dimers involving differential association with c-Jun, JunB, and/or JunD. Based on our results, AP-1 is likely to play a prominent role in transactivation of proinflammatory cytokines by VT.

IL-1β, IL-6, IL-8, TNF-α, M-CSF, and IP-10 expression are regulated, in part, by C/EBP (Akira and Kishimoto, 1997; Koj, 1996; Ohmori and Hamilton, 1994; Pope *et al*., 1994). C/EBP family members are derived from myeloid lineage and impact monocyte/macrophage differentiation (Valledor *et al*., 1998). The C/EBP family contains several family members (Akira *et al*., 1992). These include C/EBPα, which is found in undifferentiated myeloid cells, as well as C/EBPβ (nuclear factor IL-6 [NF-IL6]) and C/EBPδ that are mainly expressed in macrophages (Hanson, 1998). C/EBP proteins dimerize with other C/EBP proteins in a cell differentiation state- and tissue-specific manner, thus facilitating the differential regulation of hematopoiesis target genes (Yamanaka *et al*., 1998). C/EBPβ was specifically found to be affected by VT. This protein translocates into the nucleus following phosphorylation. The capacity of VT to sequentially upregulate and downregulate formation of at least two complexes associated with C/EBPβ might mediate initial increases in proinflammatory cytokines and other inflammatory genes, whereas disappearance of these complexes might facilitate the eventual downregulation of their expression.

CREB/ATF transcription factors act through binding to the cAMP responsive element (CRE) palindromic octanucleotide,

TGACGTCA (Mayr and Montminy, 2001). CREB-1, CREB-2, ATF-1, and ATF-2 are the best-characterized members of this family. Although the CREB/ATF family shares highly related COOH terminal leucine zipper dimerization and basic DNA binding domains, they are highly divergent in their amino terminal domains (Meyer and Habener, 1993). It was notable that unlike AP-1 and C/EBP, the CREB binding response to VT was multiphasic, with a marked depletion at 0.5 h followed by upregulation at 1.5 and 4 h. This observation may relate to differential regulation by MAPKs. Even though CREB/ATF proteins bind CREs in their homodimeric form, they sometimes also bind as heterodimers, both within the CREB/ATF family and with members of the AP-1 and C/EBP families. These latter hybrid complexes exhibit different specificities for AP-1, C/EBP, and CREB motifs. The interaction of CREB with these other factors may also explain the existence of at least four CREB bands as well as multiple bands EMSAs for AP-1 in this current study. Further investigation into identification of these multiple bands is warranted, using gel conditions that provide higher band resolution as well as use of multiple antibodies during supershifts.

The NF-κB transcriptional activator family responds to numerous stimulatory agents that include LPS, phorbol esters, IL-1, IL-2, TNF-α. NF-κB regulates many genes involved in acute phase responses and inflammation, such as GM-CSF, G-CSF, TNF-α, IL-1β, IL-6, IL-2, IL-8, and NO synthase (Akira and Kishimoto, 1997; Baldwin, 1996). This factor was first identified as a protein complex consisting of a 65 kDa DNA (p65 or RelA) binding subunit and an associated 50 kDa protein (p50). p65 is functionally related to c-Rel (p75). Heterodimers or homodimers of these proteins are normally inactive in the cytoplasm, and are associated with an inhibitory factor, IκB. After stimulation, IκB is phosphorylated, ubiquinated, and degraded releasing the NF-κB dimer which translocates into the nucleus as an active form and can activate (via p65, c-Rel, and Rel B heterodimers with p50) or suppress (via p50 homodimer) transcription of various responsive genes (Baldwin, 1996). The results found here suggest that both a c-Rel-p50 heterodimer and p50 homodimer may be upregulated in spleen late in the time period tested. This latter protein may have attenuating properties that contributes to cytokine downregulation by 6 h.

EMSA is based on the principal that proteins of differing size, molecular weight, and charge will exhibit different electrophoretic mobility in non-denaturing gels (Laniel *et al*., 2001). Interaction with DNA-binding proteins will cause DNA to characteristically migrate more slowly than free DNA. Clearly, EMSA is an *in vitro* assay and regulation of genes *in vivo* within the context of chromatin is undoubtedly much more complex, involving interactions of multiple factors. Our results represent the first step in elucidating this complex system *in vivo* that can be used as a basis for further detailed analyses. Any interpretation of the EMSA results or design of future follow-up studies must recognize that formation and electrophoretic mobility of DNA protein complexes are modulated by DNA sequence, molecular weights of the protein and the DNA, ionic strength and the pH of the binding and electrophoresis buffers, concentration of gel matrix, and temperatures used for binding reaction and electrophoresis (Taylor *et al*., 1994). For the purposes of simplicity, a uniform buffer concentration and assay condition for all four transcription-factor families was employed in the current study. Also, consensus sequences were used here because no single gene was targeted. Another consideration is that EMSA was applied to crude nuclear extracts from the spleen. A possible complication with using animal tissues can result from the presence of numerous enzymes that can degrade or modify the transcription factors (Laniel *et al*., 2001). We dealt with this issue by including both protease and phosphatase inhibitors in the extraction buffer.

Taken together, the patterns observed here for MAPK phosphorylation and differential activation/inhibition of binding activity within each transcription factor family are consistent with the known half-life of this toxin in tissue and the transient nature of VT-induced proinflammatory cytokine and other inflammation-associated genes. The data presented herein suggest the feasibility of examining the *in vivo* effects of VT and other toxicants on kinase signaling pathways and on activation of individual transcription factors within the context of other factors. One limitation of this study is that, for some endpoints, earlier submaximal responses were not observed at time points chosen. Thus future studies should examine additional time points to more precisely pinpoint peak MAPK and transcription factor responses. Although use of crude spleen homogenates precluded identification of specific cell phenotypes in which MAPK, transcription factor and cytokine effects occurred, future studies can address this issue by using immunohistochemistry to detect phosphorylated MAPKs, transcription factor activation/ sequestration, and cytokine mRNA expression in the context of cellular phenotype. These data are significant to human and animal health, because the induction of cytokines by VT and inflammation-associated genes may play a role in acute gastroenteritis as well as in chronic illness resulting from immune dysfunction. Furthermore, the results offer a venue for understanding how upstream events such as MAPK activation relate to regulation of gene expression in intact mice. The precise linkages might be explored *in vivo* using pharmacologic inhibitors for specific MAPKs in conjunction with the new *in vivo* imaging strategy of Carlson *et al*. (2002) that employs transgenic mice, which express luciferase under control of transcription factors.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grants ES-03358 (J.J.P.) and DK58833 (J.J.P.). We thank Alexa Smolinski for assistance with the cytokine mRNA analysis and Mary Rosner for manuscript preparation.

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