Repression of Protein Kinase C and Stimulation of Cyclic AMP Response Elements by Fumonisin, a Fungal Encoded Toxin Which Is a Carcinogen

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Abstract

Fusarium moniliforme (FM) is a major fungal pathogen of corn and is involved with stalk rot disease. FM is widely spread throughout the world, including the United States. Most strains of FM produce several mycotoxins, the most prominent of which is called fumonisin. Recent epidemiological studies indicated that ingestion of fumonisin correlates with a higher incidence of esophageal cancer in Southern and Northern Africa and China. Furthermore, fumonisin causes a neurodegenerative disease in horses, induces hepatic cancer in rats, and induces pulmonary edema in swine. Considering that high levels of fumonisin have been detected in the food chain, the presence of which is called fumonisin bears a remarkable structural similarity to sphingosine. Sphingomyelin has also been hypothesized to initiate a signal transduction cycle, with ceramide being generated as a second messenger (19). Although the effectors of this cycle have not been identified, they may include ceramide-activated protein kinases (20) or phosphatases (21).

PKC, a serine/threonine kinase, is central to one of the major mammalian signal transduction pathways and is activated upon external stimulation of cells by various ligands including hormones, neurotransmitters, and growth factors (22). These external signals induce the hydrolysis of membrane-associated inositol phospholipids generating two second messengers, inositol triphosphate and DAG, an endogenous activator of PKC. Phorbol esters, which are capable of tumor promotion, can substitute for DAG in enzyme activation, but unlike DAG, phorbol esters are not rapidly metabolized and thus are useful for in vitro analysis (23). PKC in mammals is comprised of at least 12 distinct polypeptides or isoforms (reviewed in Ref. 24). PKC plays a role in differentiation, cell growth, and transcription (25, 26), and there is growing evidence that specific PKC isoforms orchestrate these processes. This hypothesis is supported by the finding that marked tissue differences exist among the PKC isotypes. Moreover, the subcellular distribution of PKC isoforms changes dramatically when the cell is activated, and specific PKC isoforms have substrate selectivity. Physiologically, activation of PKC is regulated by DAG, which leads to a rapid translocation from the cytosol to the membrane. Since PKC can be found in the nucleus (27), PKC can directly phosphorylate nuclear proteins. A primary target of PKC-dependent signal transduction is a cis-acting transcriptional motif (AP-1; reviewed in Refs. 24 and 28). A family of transcription factors, including the gene products of the proto-oncogenes Fos and Jun, specifically bind to the AP-1 site, and their activation occurs by a PKC-dependent mechanism. Thus, alterations in PKC activity would have a profound effect on cellular processes.

Two major signal transduction pathways are: (a) PKC-dependent signal transduction (reviewed in Ref. 24); and (b) PKA-dependent signal transduction (reviewed in Refs. 29–32). PKA-dependent signal transduction is a cAMP-dependent mechanism by which fumonisin elicits its carcinogenic effect. Our studies indicate that fumonisin represses expression of protein kinase C activity, it appears that cyclic AMP response element activation was independent of protein kinase A. It is hypothesized that the ability of fumonisin to alter signal transduction pathways plays a role in carcinogenesis.

Introduction

FM is a fungal pathogen of corn and is found in all parts of the world, including the United States. FM is a causative agent of stalk rot disease, an economically important disease of corn (1–4). Most strains of this fungus produce a variety of mycotoxins, the major class of which are called fumonisin. Fumonisin is comprised of at least four closely related structural analogues, fumonisin Bt_4, which resemble sphingolipids (5). Only B1 and B2 are of toxicological significance because B3 and B4 plus the two A toxins are present at very low concentrations on plants. Furthermore, B3, B4, A1, and A2 toxins exhibited no cancer-initiating activity when tested at concentrations where B1 and B2 were active (6). If rats are fed a diet that contains 50 mg/Kg fumonisin B1, primary hepatocellular carcinoma occurs in two of three of the animals (6–9). Fumonisin also increases the incidence of esophageal cancer in Africa and China (10), induces equine leukoencephalomalacia or hepatotoxicity (11), and porcine pulmonary edema (12). Fumonisin can be detected on healthy plants (13, 14), suggesting that the seriousness of fumonisin in the food chain is underestimated. For example, fumonisin is present in substantial amounts, up to 2.7 parts/million, in 74% of U.S. corn-based food products. Taken together, these observations suggest that fumonisin may be a health risk to humans.

Structurally, fumonisin resembles sphinganine, which is an intermediate in the biosynthesis of the sphingosine backbone of more complex sphingolipids such as ceramides, sphingomyelin, cerebrosides, gangliosides, and sulfatides (Fig. 1A; reviewed in Ref. 15). Several studies have demonstrated that the addition of fumonisin to cultured cells or to the diet of rats inhibits sphingolipid biosynthesis (15–17). An important site of inhibition is the reaction catalyzed by sphingosine N-acetyltransferase (ceramide synthase). Sphingolipids have been implicated as playing a role in cell contact, growth, and differentiation. Since fumonisin bears a remarkable structural similarity to sphingosine (Fig. 1A; Ref. 17) and sphingosine is a potent inhibitor of PKC in vitro (18), it is possible that fumonisin alters PKC activity. Sphingomyelin has also been hypothesized to initiate a signal transduction cycle, with ceramide being generated as a second messenger (19). Although the effectors of this cycle have not been identified, they may include ceramide-activated protein kinases (20) or phosphatases (21).

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3 The abbreviations used are: FM, Fusarium moniliforme; PKC, protein kinase C; DAG, diacylglycerol; PKA, protein kinase A; CRE, cAMP response element; CREB, CRE-binding proteins; CMF, calcium/magnesium-free; CAT, chloramphenicol acetyltransferase; PBS-T, PBS tween.
FUMONISIN REPRESSION OF PKC AND CAMP STIMULATION

Results

Repression of PKC Activity by Fumonisin. Since fumonisin structurally resembles a potent PKC inhibitor, sphingosine (Refs. 17 and 18; Fig. 1A), the effect fumonisin has on PKC activity was measured in CV-1 cells. When CV-1 cells were treated for 3 h with 5 μM fumonisin, PKC activity decreased approximately 2-fold relative to controls (Fig. 1B). Repression of PKC activity was also evident after cells were treated with 1 or 5 μM fumonisin for 16 h. Repression was dose dependent since 0.01 μM had a negligible effect and 0.1 μM only inhibited 15% after 3 h treatment. In contrast, PKA levels were not affected by any fumonisin concentration used in these studies. These studies indicated that fumonisin inhibited PKC activity in CV-1 cells after only 3 h treatment and repression required at least 1 μM fumonisin.

The effect fumonisin has on steady-state levels of PKC was assessed by Western blot analysis. A panel of antibodies directed against the major isoforms of PKC (α, β, and γ) were used for these studies. A M120,000 protein was consistently repressed after CV-1 cells were treated with fumonisin (1 or 5 μM) for 3 or 16 h (Fig. 2). In addition, two proteins migrating with an apparent molecular weight of 70,000 and 83,000 were repressed when cells were treated with 1 μM fumonisin for 3 h. However, levels of a protein migrating with an apparent molecular weight of 65,000 was not altered by fumonisin. Since there are at least 12 different isoforms of PKC in mammals (reviewed in Ref. 24), isotype-specific antibodies were used to identify which PKC isoforms were repressed by fumonisin. A PKC α antibody recognized a M120,000 protein which was repressed by fumonisin (Fig. 3A). A PKC θ antibody recognized a M63,000 protein which was repressed by fumonisin (Fig. 3B). Antibodies

Materials and Methods

Fumonisin. Two sources of fumonisin B1 were used in these studies: Sigma (>98% pure); or preparations obtained from Dr. R. D. Plattner (USDA, Peoria, IL; >90% pure). Identical results were obtained with both preparations. Fumonisin was dissolved in CMF PBS [80 mm Na2HPO4, 20 mm NaH2PO4, and 100 mM NaCl (pH 7.5)] and stored at 4°C in the dark.

Cells and Media. CV-1 cells (African green monkey kidney cells) were obtained from Robert Su (NIH, Bethesda, MD). Cells were grown in Earle's modified Eagle's medium supplemented with 5% fetal bovine serum. All media contained penicillin (10 units/ml) and streptomycin (100 mg/ml).

Measurement of CAT Activity in Transfected Cells. Cells were transfected with 10 μg of the designated DNA using CaPO4 precipitation as described previously (33-37). A DMSO or glyceral shock was not used because this treatment altered 12-O-tetradecanoylphorbol-13-acetate response in CAT assays (33, 34). Forty h posttransfection, CAT activity was measured as described previously (35-37). Amounts of radioactivity associated with the protein which was repressed by fumonisin (Fig. 3B). Antibodies

Measurement of PKC and PKA Activity. PKC activity and PKA activity was conducted as described previously (39).

Western Blot Analysis. CV-1 cells, normal or fumonisin treated, were washed in CMF-PBS and scraped with a rubber policeman. Cells were pelleted by centrifuging (2000 RPM for 10 min). The cell pellet was resuspended in CMF-PBS. Total proteins were extracted from cells, and 50 μg protein were electrophoresed in 8% SDS polyacrylamide gels. Protein concentrations were measured by the method of Bradford (40). Proteins were electrochemically transferred to nitrocellulose (Hybond ECL; Amersham) according to the manufacturer's recommendations. Filters were blocked with PBS-T for 1 h at room temperature with shaking. Filters were incubated with a mixture of PKC antibodies (α, β, and γ) for 1 h at room temperature in PBS-T. Following washing, a horseradish peroxidase-labeled secondary antibody was added, and the membrane was incubated for 1 h at room temperature. The filter was subsequently washed with PBS-T. Bands were visualized by chemiluminescence using ECL reagents (Amersham).
directed against PKC B1, B2, and γ did not detect proteins which had lower steady-state levels after fumonisin treatment (data not shown).

In summary, these studies identified specific isoforms of PKC, α and θ, that were repressed by fumonisin.

Analysis of Transcription after Fumonisin Treatment. To address the effect fumonisin has on transcription, CV-1 cells were transfected with promoter constructs containing 5 AP-1 binding sites (5x col/cat TRE) or a single CRE (CRE/cat), and promoter activity was measured after fumonisin treatment. Promoter activity of 5x col/cat TRE was reduced more than 10-fold when CV-1 cells were treated with 5 or 10 μM fumonisin for 3 h (Fig. 4B). However, promoter activity of 5x col/cat TRE was reduced only 2-fold after 16 h treatment (Fig. 4A). One copy of an AP-1 binding site linked to the thymidine kinase promoter was also repressed by fumonisin (data not shown). Previous studies by us (33, 34, 37) and others (41, 42) demonstrated that a phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, stimulates promoter activity of 5x col/cat TRE, lending support to the hypothesis that fumonisin antagonizes PKC activity. In contrast to 5x col/cat TRE, promoter activity of CRE/cat was stimulated approximately 10-fold after treatment with 1 or 5 μM fumonisin for 3 h (Fig. 4B). Treatment for 16 h eliminated the stimulatory effect of fumonisin. Promoter activity of a construct lacking the 5 AP-1 binding sites or the CRE (pBLcat/4) was not affected by fumonisin treatment. These results indicated fumonisin had differential effects on a CRE or AP-1 binding site linked to a thymidine kinase promoter.

Discussion

In this study, we examined whether there was a link between fumonisin B1, PKC, and downstream targets of PKC in an established cell line, CV-1. Since the structure of fumonisin B1 is similar to the well-known PKC inhibitor, sphingosine, we hypothesized that PKC is a target of fumonisin B1. The effect fumonisin B1 has on AP-1-dependent transcription was also examined because AP-1-dependent transcription is regulated by PKC (24, 28). It was evident from these studies that fumonisin B1 decreased steady-state levels of specific PKC isoforms and transiently repressed AP-1-dependent transcription. In contrast, CRE-dependent transcription was stimulated by fumonisin B1, but stimulation appeared to occur in a PKA-independent fashion. It is hypothesized that the ability of fumonisin to act as a carcinogen or toxin is linked to its ability to repress specific PKC isoforms and consequently alter signal transduction pathways.

Although fumonisin did not eliminate PKC activity, repression was reproducible and occurred in a dose-dependent fashion. The concentrations required to observe PKC repression was 1–5 μM or 0.72–3.6 mg/liter. If rats are fed a diet that contains 50 mg/Kg fumonisin B1, primary hepatocellular carcinoma occurs in two of three of the animals (6–9). Fumonisin B1 disrupts sphingolipid metabolism and stimulates DNA synthesis at concentrations ranging between 20–50 μM (43). The concentrations of fumonisin necessary to observe PKC repression were low relative to other in vivo and in vitro studies and thus appear to be physiologically relevant. It was not surprising that significant levels of PKC activity were detected after fumonisin treatment since multiple isoforms of PKC were apparently expressed in CV-1 cells but not all isoforms were repressed by fumonisin (Fig. 2). After CV-1 cells were treated with fumonisin for 3 or 16 h, a M, 120,000 band was repressed, which appears to be PKC α. Although most PKC isoforms have a molecular weight less than M, 100,000, a recent study identified a novel PKC isoform, PKKu, which has a molecular weight of 115,000 (44). Repression of two PKC isoforms, 72,000 and 83,000 proteins, was detected after 3 h of fumonisin treatment but not 16 h (Fig. 2), suggesting that repression occurs in a transient manner. However, isotype-specific antibodies failed to recognize these proteins. Western blots probed with all of the available isotype antibodies detected a band migrating with an apparent molecular weight of 115,000, which was not affected by fumonisin treatment (Fig. 2). However, a PKC θ-specific antibody detected a M, 63,000 protein which had lower steady-state levels after fumonisin treatment.
Fumonisin repression of PKC and cAMP stimulation

(Fig. 3B). It is known that multiple PKC isoforms migrate with an apparent molecular weight between 60,000 and 70,000 (reviewed in Ref. 24). Thus, it is likely that more than one isoform, with molecular weights between 60,000 and 65,000, was detected when Western blots were probed with a mixture of PKC antibodies.

There is mounting evidence that specific isoforms of PKC play distinct roles in regulating cellular proliferation or differentiation (reviewed in Refs. 24 and 45). In K562 erythroleukemia cells, expression of PKC B2 correlates with proliferative capacity (26). In contrast, expression of PKC α correlates with induction of megakaryocytic differentiation and cessation of growth. PKC is also intimately involved in the normal processes and survival of neurons (reviewed in Ref. 45). Expression of c-jun and c-fos is stimulated after cells are treated with growth stimulating factors: serum, epidermal growth factor, transforming growth factor α, or platelet-derived growth factor (41, 46–48). Nerve growth factor also induces c-jun and c-fos in PC12 cells, but this treatment arrests growth and induces neuronal differentiation (49). It is likely that, in some tissue, induction of PKC and the AP-1 complex leads to differentiation, whereas in other cell types, it stimulates proliferation. Since fumonisin represses PKC activity and specific isoforms of PKC (Figs. 2–4), it is tempting to speculate that repression of PKC isoforms is necessary for its carcinogenic potential. This hypothesis is supported by the fact that fumonisin B1 is not a potent carcinogen in a standard Ames test (8) but can induce proliferation of Swiss 3T3 cells in conjunction with insulin (43).

AP-1-dependent transcription is regulated by numerous diverse mechanisms involving the jun and fos family of transcription factors (reviewed in Refs. 24 and 28). Stimulation of PKC results in the dephosphorylation of specific residues proximal to the DNA binding domain of c-jun. Specific PKC isoforms (α, β, γ, but not ε) phosphorylate glycogen synthase kinase-3B, thus preventing glycogen synthase kinase-3B from phosphorylating the DNA binding domain of c-jun (50). Conversely, phosphorylation of specific amino acids at the NH2 terminus of c-jun by a distant relative of MAP kinase, JNK1, activates c-jun (51, 52). At least two proteins which bind c-jun repress c-jun DNA binding and activation of AP-1 dependent transcription: IP-1 (53) and Jif-1 (54). In addition to fos, it is clear that CREB, specific members of the ATF family, and NFKB bind to c-jun and regulate AP-1 transcription (reviewed in Refs. 29–32, 55, 56). Two other members of the jun family, JunB and JunD, can also dimerize with c-jun and alter AP-1-dependent transcription (57, 58). The ability of fumonisin to inhibit PKC activity would, therefore, have a negative effect on AP-1-dependent transcription. However, repression of AP-1-dependent transcription might not be limited to PKC repression.

The observation that fumonisin activated a CRE linked to a thymidine kinase promoter in transient transfection assays but had no effect on PKA activity (Figs. 1 and 4) suggested that transcriptional activation was independent of PKA. Although CREB is the major activator of CRE-dependent transcription and is primarily activated by PKA, other transcription factors can positively regulate CRE-dependent transcription (reviewed in Refs. 29–32). For example, members of the ATF family can bind and activate a CRE. CREB can also dimerize with members of the ATF family and jun family members. Since a member of the Jun family (JunB) activates a CRE but represses transcription of genes which carry AP-1 binding sites (57, 58), it is possible that fumonisin activates junB. The finding that calcium/calcmodulin-dependent protein kinases have the potential to phosphorylate and activate CREB (59) also suggested that fumonisin may...
activate other protein kinases which positively regulate CRE-dependent transcription. Experiments designed to determine how fumonisin activates a CRE but represses AP-1 are currently in progress.

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References

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