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Aflatoxin B_1 Alters the Expression of p53 in Cytochrome P450-Expressing Human Lung Cells

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Aflatoxin B_1 (AFB_1) is a potent dietary hepatocarcinogen in animals and probably in humans. Mutations (and altered expression) of the tumor suppresser gene *p53* have been observed in liver tumors from patients exposed to high dietary AFB_1. Inhalation of AFB_1-laden grain dusts has been associated with an increased incidence of lung cancer in humans as well. We examined the effects of low concentrations of AFB_1 on the expression of p53 and MDM2 in human bronchial epithelial cells (BEAS-2B) transfected with cDNA for either cytochrome P450 (CYP) 1A2 (B-CMV1A2) or CYP 3A4 (B3A4), two isozymes that are responsible for AFB_1 activation in human liver and possibly the lung. Untreated B-CMV1A2 and B3A4 cells constitutively expressed p53. Exposure to a range (0.015–15 μM for 30 min) of AFB_1 concentrations caused a concentration-dependent decline in p53 expression in B-CMV1A2 cells, and to a lesser extent, in B3A4 cells. The AFB_1-mediated decrease in p53 continued for at least 12 h after 30-min exposures to 1.5 μM AFB_1. Mirroring the decrease in p53 expression was a concentration-dependent increase in the expression of the 76-kDa MDM2 isoform in B-CMV1A2 and B-3A4 cells. Interestingly, AFB_1 did not induce DNA laddering, an indicator of apoptotic cell death, but proteolytic activation of caspase-3 was detected in AFB_1-treated B-CVM1A2 cells. In total, these data show that low, environmentally-relevant concentrations of AFB_1 alter the expression of p53 and MDM2 in these human lung cells, and that cells that stably express CYP 1A2 were more susceptible to this effect than nontransfected, or 3A4-expressing cells.

Aflatoxin B_1 (AFB_1) is a potent immunotoxicant and hepatocarcinogen in animals and probably in humans (Bondy and Pestka, 2000; Klein *et al*., 2000). The liver is the primary target organ, because AFB_1 requires metabolic activation to form the reputed carcinogenic species AFB_1-8,9-epoxide (AFBO) (Mace *et al*., 1994, 1997), but other organs are also affected by AFB_1 exposures (Ball and Coulombe, 1991; Ball *et al*., 1995; Coulombe *et al*., 1991; Imaoka *et al*., 1992; Kato *et al*., 1994; Kelly *et al*., 1997; Liu *et al*., 1990, 1993; Liu and Massey, 1992). In human liver, CYPs 1A2 and 3A4 have been shown to be the principle enzymes responsible for AFB_1 activation (Ramsdell *et al*., 1991; Shimada and Guengerich, 1989) and have also been detected in human lung tissues and cultured lung cells (Mace *et al*., 1998; Van Vleet *et al*., 2001; Wei *et al*., 2001).

Inhalation of respirable AFB_1-contaminated grain dusts may pose a cancer hazard to susceptible individuals in certain agricultural occupations (Hayes *et al*., 1984). AFB_1 is activated to AFBO in animal (Ball *et al*., 1995; Daniels *et al*., 1990; Daniels and Massey, 1992; Imaoka *et al*., 1992; Liu *et al*., 1990; Liu and Massey, 1992) and in human pulmonary tissues (Autrup *et al*., 1979; Donnelly *et al*., 1996; Kelly *et al*., 1997).

The tumor suppressor gene *p53* is the most commonly mutated gene in human cancers, with mutations in approximately 50% of all human cancers (Chang *et al*., 1993; Kew, 1992; May and May, 1995; Nigro *et al*., 1989). Dietary AFB_1 exposure has been linked to G→T transversions at codon 249 of *p53* in human primary hepatocellular carcinomas (PHC) (Cerutti *et al*., 1994; Hainaut and Vahakangas, 1997; Soini *et al*., 1996) and in cultured human hepatocytes exposed to AFB_1 (Aguilar *et al*., 1993; Mace *et al*., 1997). Therefore, this mutation may inactivate p53, leading to AFB_1-induced liver cancers (Hollstein *et al*., 1993; Lasky and Magder, 1997). It seems reasonable that a similar mutation may be produced by AFB_1 in the lung.

After cellular DNA damage, p53 protein is phosphorylated by DNA-dependent protein kinase, which arrests the cell cycle in the G1 phase, thus preventing mitosis and allowing the repair of damaged sequences (Kastan *et al*., 1992). Thus, by preventing the proliferation of damaged cells, p53 acts to protect the integrity of the genome (Lane, 1992). Induction of p53 leads to increased MDM2 expression, which eventually inhibits p53 expression via a negative-feedback mechanism (Pochampally *et al*., 1998). To induce MDM2 expression, p53 acts as a transcriptional element on the MDM2-P2 promoter

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(Hesketh, 1997; Ralhan *et al*., 2000), but MDM2 induction is at least partly also due to mRNA stabilization (Hsing *et al*., 2000). To date, several MDM2 proteins have been identified (90, 76, 60, 46, and 35 kDa) (Maxwell, 1994; Mendrysa *et al*., 2001; Ralhan *et al*., 2000), which are produced by differential splicing of the mRNA transcript and caspase-3-mediated cleavage of the 90-kDa isoform (Chen *et al*., 1997; Maxwell, 1994; Mendrysa *et al*., 2001) and by internal initiation at codon 50 of the *mdm2* mRNA (Saucedo *et al*., 1999).

The proteolytic activation of caspase-3 and DNA ladder formation are key steps in the apoptotic cascade (Maruyama *et al*., 2001) with caspase-3 activation regarded as a primary mechanism of apoptosis (He *et al*., 2003). Caspase-3-like activity has been implicated in the processing of MDM2 to a form that stabilizes p53 (Pochampally *et al*., 1999). Caspase-3 activation can be detected using Western immunoblotting to demonstrate proteolytic cleavage of the procaspase-3 protein (35 kDa) to the largest (17 kDa) proteolytic fragment (Erhardt *et al.*, 2001).

The BEAS-2B cell line, a simian virus 40 (SV-40) large T antigen immortalized version of normal human bronchial epithelial (NHBE) cells, is an *in vitro* model for the study of the human lung toxicity (Reddel *et al*., 1988). Infection of normal cells with SV-40 interferes with p53 function, leading to immortalization (Carnero *et al.*, 2000; Hsieh *et al.*, 2000; Peterson *et al.*, 1995; Porras *et al.*, 1999). The SV-40 viral genome codes for the large T antigen, which binds p53, inhibiting its normal growth arrest and cell cycling functions (Levresse *et al*., 1998; Peterson *et al*., 1995; Porras *et al*., 1999).

We recently demonstrated that BEAS-2B cells transfected to stably express CYP 1A2 (BCMV-1A2) and 3A4 (B3A4) activate AFB_1 to cytotoxic and DNA-alkylating species (Van Vleet *et al*., 2002a,b). Because AFB_1 affects p53 expression in human liver cancer (Barton *et al*., 1991), we wished to determine if AFB_1 would affect this tumor suppressor system in these immortalized lung cells. Our data indicate that AFB_1 perturbs the expression of p53 and related proteins in these cells when critical CYPs are expressed. At the lowest concentrations studied, CYP 1A2-expressing cells were affected to a greater extent than those expressing 3A4, in support of previous results showing that expression of the former isoform may be more relevant to AFB_1 toxicity. Despite compromised p53 activity, exposure to AFB_1 activates caspase-3 in BCMV1A2 cells.

MATERIALS AND METHODS

Chemicals and reagents. LHC-8, LHC-9, LHC Basal, epinephrine, retinoic acid, and bovine serum albumin (BSA) stock were obtained from BioWhittaker (Rockville, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Bovine fibronectin, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), glycerol, AFB_1, trypsin inhibitor, aminosalicylate, β-mercaptoethanol, pyronin-Y, staurosporine, caffeine, and Coomassie blue were purchased from Sigma (St. Louis, MO). Collagen was a product of Collagen Corp. (Fremont, CA). CHAPS Cell Extraction buffer, primary polyclonal rabbit anti-human caspase-3 antibody, primary polyclonal rabbit p53 antibody used with the BEAS-2B cells, and secondary antirabbit IgG were purchased from Cell Signaling, (Beverly, MA). Primary monoclonal mouse p53 and MDM2 used with the BCMV-1A2, B3A4, and NHBE cells were from Calbiochem (San Diego, CA). Secondary (goat-anti-mouse) antibody was from Bio-Rad (Hercules, CA). ECL chemiluminescent reagent was from Amersham (Piscataway, NJ). Supersignal West Femto chemiluminescent substrate was from Fisher Scientific, (Pittsburg, PA). BEAS-2B, B-CMV1A2, and B3A4 cells were a generous gift from Dr. Katherine Macé (Nestle Research Centre; Lausanne, Switzerland). Normal human bronchial epithelial (NHBE) cells were purchased from BioWhittaker (San Diego, CA).

Cell culture. BEAS-2B, B-CMV1A2, and B3A4 cells were cultured as previously described (Van Vleet *et al*., 2002a) for all experiments excluding caspase-3 activation and basal p53 expression in BEAS-2B wherein flasks were not coated with a plate coat consisting of 5 mg bovine fibronectin, 5 ml collagen, 50 ml BSA stock, and 500 ml LHC Basal. NHBE cells were cultured as previously described (Van Vleet *et al*., 2001).

Preparation of cell lysates. Cells were seeded at a density of 9.5×10^5 cells/T75 flask and cultured for 48 h. For time-course studies, cultures were then exposed to 1.5 μM AFB_1 for 30 min, after which flasks were washed with PBS, and then fresh media was added to cultures. Cells were harvested via trypsinization, at various time intervals thereafter (1, 2, 4, 6, 9, and 12 h). To determine the effects of a range of AFB_1 concentrations on p53 expression, cells were exposed to AFB_1 (0.015–15 μM) for 30 min, and flasks were then washed with PBS. The cells were cultured for 6 h in fresh media before they were harvested. Next, cells from each T75 flask were resuspended in 1 ml of LHC-9. The cell density was determined (Counter Model F^N; Beckman-Coulter Fullerton, CA), and 0.5 ml of the cell suspension was centrifuged to collect cells. After removing the supernatant, cell lysing buffer (2% sodium dodecyl sulfate (SDS), 12% aminosalicylate, 2% NaCl, and 12% 2-butanol) was added to the pellet at a concentration of 100,000 cells/20 μl. Samples were stored at −80°C until separated by SDS–PAGE. Control groups were also run at each time point (time-course study), or at 6 h after exposure to AFB_1 (concentration range study) for 30 min.

Measurement of p53 and MDM2 expression. Cell lysates (20 μl) were heated in sample buffer (10% SDS, 0.5M Tris-HCl, 20% glycerol, 10% β-mercaptoethanol, 0.1% pyronin-Y; 30 μl; total vol = 50 μl) to 70°C for 5 min and loaded into 10–15% SDS-PAGE gels (14 × 11 × 0.1 cm), with duplicate lanes, then electrophoresed for 8 h at 125 V. One-half of each gel was transferred to a Nitrobind nitrocellulose transfer membrane (Micron Separations, Inc.) using a semi-dry blotter (Buchler, Kansas City, MO). The other half of the gel was stained with Coomassie blue for molecular weight analysis. Molecular weight markers served as negative controls for nonspecific binding of antibodies to protein in the immunostained gel portions, and for molecular weight approximations of the Coomassie-stained gel halves. Nitrocellulose membranes were immunostained using the primary antibody (1:5000) in High Salt Tween (HST) blocking buffer (10 mM Tris, 1 M NaCl 0.5% Tween 20, pH 7.4). Membranes were washed with HST, Tris-buffered saline (TBS) (10 mM Tris and 140 mM NaCl, pH 7.4), and TBS-Tween (TBS with 0.1% Tween 20) as described previously (Klein *et al.*, 2000). Secondary antibodies were also diluted in HST (1:2000). Proteins were detected by chemiluminescence generated by horseradish peroxidase-conjugated secondary antibody, using ECL reagent as a substrate, and quantified using a Nucleovision 920 chemiluminescence imaging workstation (Nucleotech Corp., Hayward, CA).

The following method was employed exclusively for examining p53 expression in BEAS-2B cells. Cells were grown to approximately 80% confluence and harvested via trypsinization. Cells were pelleted and resuspended in 100 μl of CHAPS (50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM PMSF) cell extract buffer and subjected to five freeze–thaw cycles. The cell lysate was then centrifuged at 13,000 rpm (16,060 × *g*) for 5 min, and the supernatant was retained and frozen at −80°C. Sample protein was measured using the Quick Start Bradford Protein assay kit

(Bio-Rad, Hercules CA). Aliquots of the supernatant were diluted 1:1 with SDS sample buffer (2% SDS, 50 mM dithiothreitol (DTT), 0.01% bromophenyl blue, 10% glycerol), boiled for 5 min, and loaded onto 4–15% SDS/Tris–HCl mini acrylamide gradient gels (Bio-Rad Labs, Hercules, CA) at 15 μg protein per well. P53 standard (Oncogene, Boston, MA) was loaded at 10 μl per well. Samples were electrophoresed for 45 min at 200 V on a Bio-Rad Mini-Protean 3 Cell electrophoresis unit (Bio-Rad Labs, Hercules, CA). Gels were transferred at 100 V for 1 h to nitrocellulose transfer membranes (GE Osmonics, Minnetonka, MN) using the Bio-Rad Mini-Protean 3 Cell electrophoresis unit. Nitrocellulose membranes were washed in TBS for 5 min and then incubated in blocking buffer (TBS with 0.1% Tween 20 and 5% nonfat dry milk). Membranes were again washed in TBS-Tween (TBS with 0.1% Tween 20) three times for 5 min each, and immuno-stained overnight (at 4°C) with the primary antibody (1:1000) in 5% BSA, 1× TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight. Membranes were then washed with TBS-Tween three times. Secondary antibodies were diluted in blocking buffer (1:2000 and 1:1000) and incubated with the membrane at room temperature for 1 h. Proteins were detected by chemiluminescence generated by horseradish peroxidase-conjugated secondary antibody, using Supersignal West Femto chemiluminescent substrate, and captured using a Nucleovision 920 chemiluminescence imaging workstation (Nucleotech Corp., Hayward, CA).

Ladder assay for apoptosis. B-CMV1A2 cells were seeded at a density of 9.5×10^5 cells/T75 flask, and cultured for 48 h. Actively dividing cells (approx. 50% confluency) were then dosed with AFB$_1$ at various concentrations (0–15 μM) for 30 min and harvested after 6 h of culture in fresh media via trypsinization. To study the time course of apoptosis, actively dividing cells were dosed with 1.5 μM AFB$_1$ for 30 min, washed with PBS, and harvested at various time intervals (1, 2, 4, 8, 12, 16, 20, 24 h) after PBS was replaced with fresh LHC-9. Cells were then harvested, and pellets were resuspended at a concentration of 2×10^6 cells/200 μl in PBS for use in assay. DNA samples (6 μg) were added to 10× loading buffer (1% SDS, 2.5 mg/ml bromophenyl blue, 30% glycerol) and loaded into 7 × 7.5 × 1 cm 1% agarose gels in TBE (0.04 M Tris, 0.04 M boric acid, 0.01 M EDTA). Samples were electrophoresed at 4°C for 35 min at 200 V. Laddering was also examined using the Qiagen DNeasy tissue Kit (Qiagen, Valencia, CA) and the proteinase K method described by Thorburn *et al.* (2003).

Detection of caspase-3 activation. B-CMV1A2 and B3A4 cells were seeded at a density of 6.4×10^4 cells/T-75 flask and cultured for 48 h. Actively dividing cells (approx. 70% confluency) were then dosed with either AFB$_1$ (1 μM), Staurosporine (1 μM; positive control), or DMSO (20 μl; negative control) for 4 and 9 h, and harvested in ice cold PBS, via scraping. Caffeine-dosed cells were exposed to 150, 250, 350, and 450 μM concentrations for 24 h. Cells were pelleted and resuspended in 100 μl of CHAPS (50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM PMSF) cell extract buffer and subjected to five freeze–thaw cycles. The cell lysate was then centrifuged at 16,000 rpm for 5 min, and the supernatant was retained and frozen at −80°C. Sample protein was measured using the Quick Start Bradford Protein Plate reader assay kit (Bio-Rad, Hercules CA) on a Labsystems Multiskan MCC/340 (Fisher Scientific, Pittsburg, PA). Aliquots of the supernatant (25 μl) were diluted 1:1 with SDS sample buffer (2% SDS, 50 mM DTT, 0.01% bromophenyl blue, 10% glycerol), boiled for 5 min, and loaded at 0.5 μg protein per well for AFB$_1$ and 24 μg per well caffeine onto 15% SDS/Tris–HCl mini acrylamide gels (Bio-Rad Labs, Hercules, CA). Samples were electrophoresed for 45 min at 200 V on a Bio-Rad Mini-Protean 3 Cell electrophoresis unit (Bio-Rad Labs, Hercules, CA). Gels were transferred at 100 V for 1 h to nitrocellulose transfer membranes (GE Osmonics, Minnetonka, MN). Nitrocellulose membranes were washed in TBS for 5 min and then incubated in blocking buffer (TBS with 0.1% Tween 20 and 5% nonfat dry milk). Membranes were again washed in TBS-Tween (TBS with 0.1% Tween 20) three times for 5 min each, and immuno-stained overnight (at 4°C) with the primary antibody (1:1000) in blocking buffer. Membranes were then washed with TBS-Tween three times for 5 min. Secondary antibodies were also diluted in blocking buffer (1:2000 anti biotin and 1:1000 anti rabbit) and incubated with the membrane at room temperature for 1 h. Proteins were detected by chemiluminescence generated by horseradish peroxidase-conjugated secondary antibody, using Supersignal West Femto chemiluminescent substrate, and images were captured and archived.

Determination of cell viability. The IC_{50} value for caffeine toxicity in BCMV-1A2 cells was determined via MTT in a 96-well format using a Labsystems Multiskan MCC/340 (Fisher scientific, Pittsburgh PA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, St. Louis, MO) as previously described (Mosmann, 1983).

Statistical analysis and curve fitting. Groups were compared for differences using one-way ANOVA (Sigma Stat Software). A $p < 0.05$ was judged significant. Curves generated from digital densitometry analysis were fit using Sigma Plot logistics curve fitting program (SPSS, Chicago, IL).

RESULTS

All SV-40 transformed cells, BEAS-2B, B-CMV1A2, and B3A4 expressed p53 constitutively (Figs. 1A and 1B). The doublets match those found by other researchers (Matlashewski *et al.*, 1986) and those shown on the manufacturer's instructions. This is in contrast to normal human bronchial epithelial (NHBE) cells, where no constitutive p53 expression was observed (Fig. 1A).

When B-CMV1A2 and B3A4 cells were exposed to 1.5 μM AFB$_1$ for 30 min, p53 expression decreased over time compared to their respective unexposed controls (Figs. 2A and 2B). This decrease in expression continued at least 12 h, with the effect being significantly greater in B-CMV1A2 cells than in B3A4 cells at all time points (Figs. 2A, 2B, and 2C). We then determined whether the inhibitory effect on p53 expression was dependent on AFB$_1$ concentration. When exposed to a range of AFB$_1$ concentrations (0–15 μM) for 30 min, a similar decrease in p53 expression was observed in both B-CMV1A2 and B3A4

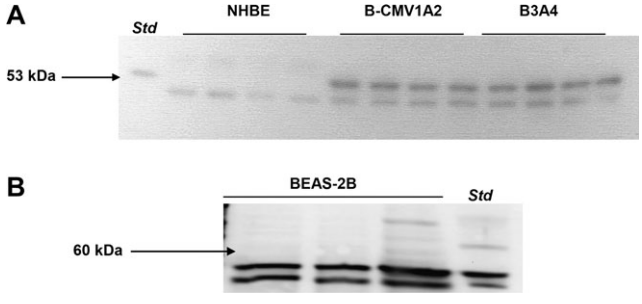

FIG. 1. Representative Western immunoblots showing constitutive p53 expression in BEAS-2B, B-CMV1A2, and B3A4 cells in contrast to that in Normal Human Bronchial Epithelial (NHBE) cells (A,B). (A) B-CMV1A2, B-3A4, and NHBE cell lysates were prepared as described in Materials and Methods (100,000 cells/20 μl), and 20 μl of the lysate was loaded into gels for analysis. Cell lysate proteins were separated with 10% polyacrylamide SDS–PAGE gels at 125 V for 8 h. (B) BEAS-2B cell lysates were prepared as described in the Materials and Methods, and 15 μg of lysate protein was loaded/well, each lane representing protein from different flasks. Proteins were separated on 4–15% SDS/Tris–HCl mini acrylamide gels at 200 V for 45 min.

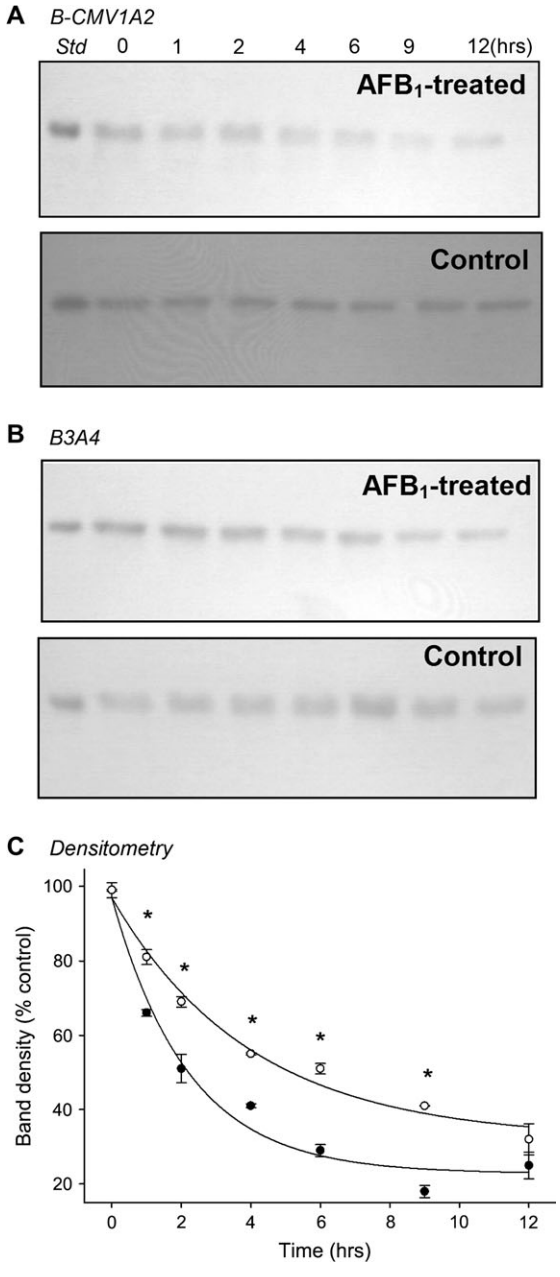

FIG. 2. Representative Western immunoblots showing a decrease in p53 expression resulting from a 30-min exposure to AFB_1 (1.5 μM) in lysates prepared from B-CMV1A2 (A) and B3A4 (B) cells. After exposure, cells were washed with PBS and cultured for 1–12 h in LHC-9 before harvest. Experimental details and cellular lysate preparation protocol are described in Materials and Methods. As described, 20 μl of cell lysate (100,000 cells/20 μl) was loaded into gels for analysis. Cell lysate proteins were separated with 10% polyacrylamide SDS–PAGE gels at 125 V for 8 h. (C) Digital densitometric analysis of Western immunoblots showing comparative p53 expression at various intervals following a 30-min exposure to 1.5 μM AFB_1 such as shown in (A,B). AFB_1 treatment in B-CMV1A2 cells (●) caused a greater decrease in p53 expression over time than that observed in B3A4 cell cultures (○). P53 standard was used, as an internal standard, to correct for slight differences in exposure times. Control densities for each time point were unchanged. Data points are the means of 3 replications (three separate experiments of three pooled flasks each). *Indicates time points significantly different between the two groups.

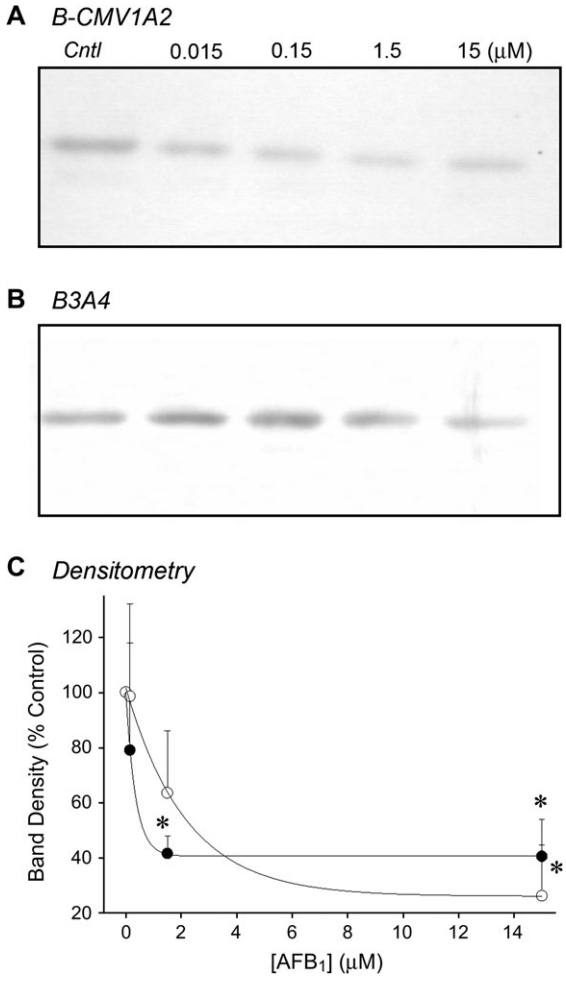

FIG. 3. Representative Western immunoblots showing that the inhibition of p53 expression by AFB_1 in B-CMV1A2 (A) and B3A4 (B) cells is AFB_1-concentration dependent. Cells were exposed to a range of AFB_1 (0.015–15 μM) for 30 min, and p53 expression was measured 6 h later. B-CMV1A2 cells had a slightly greater decrease in p53 expression than the B3A4 cells at the lower AFB_1 concentrations. Cell lysates were prepared as described in Materials and Methods (100,000 cells/20 μl), and 20 μl of the lysate was loaded into gels for analysis. Cell lysate proteins were separated with 10% polyacrylamide SDS-PAGE gels at 125 V for 8 h. (C) Graphical representations of immunoblot densitometry show the relative decreases in p53 expression in B3A4 (○) and B-CMV1A2 cells (●) 6 h after a 30-min exposure to AFB_1 at a range of concentrations (0.015–15 μM) in B-CMV1A2 and B3A4 cells. Data points represent the means of band densities, determined by digital densitometry, of three immunoblots as shown in (A,B) expressed as percent control. *Indicates concentrations significantly different from corresponding controls (0 μM AFB_1).

cell types in a concentration-dependent manner 6 h post-exposure (Figs. 3A and 3B). Thus, the inhibitory effect of AFB_1 on p53 expression was dependent on AFB_1 concentration and duration of exposure. In cultures exposed to the highest AFB_1 concentration (i.e., 15 μM), the decrease in p53 expression was greater in B3A4 than the B-CMV1A2 cells (Fig. 3C).

Because p53 expression was altered to a greater extent in B-CMV1A2 cells, we then examined the effect of AFB_1 on the

expression of MDM2 in these cells. As can be seen in Figure 4, AFB$_1$ elicited an increase in MDM2 (76 kDa) expression in B-CMV1A2 cells in both a concentration- (0.15–15 μM; 6 h after 30-min exposures) and time-dependent (0–12 h after 30-min exposures to 1.5 μM AFB$_1$) fashion (Figs. 4A and 4B). When data from Figures 3 and 4 are plotted together, the combined effect of AFB$_1$ on both p53 and MDM2 can clearly be seen in Figure 5. The AFB$_1$ concentration and time-dependent decrease in p53 expression were mirrored by a concomitant increase in MDM2 (76 kDa) expression (Figs. 5A and 5B) at each point. However, only expression of the 76-kDa isoform was consistently affected by AFB$_1$. Other MDM2 proteins—90, 60, and 35 kDa—were detected, but their levels were not affected by AFB$_1$ treatment (data not shown).

DNA ladder formation, an indicator of the onset of an irreversible stage in apoptosis, was examined in B-CMV1A2 cells. B-CMV1A2 cells were exposed to 1.5 μM at a range of post-AFB$_1$ exposure intervals (0–24 h; Fig. 6A). No ladder formation could be detected in B-CMV1A2 cells treated with AFB$_1$. Even when B-CMV1A2 cells were subjected to a 30-min exposure at a range of AFB$_1$ concentrations (0.015–15 μM; 6-h exposure), no DNA ladder formation was detected (Fig. 6B). There was no clear indication of ladder formation detected in any AFB$_1$- or staurosporine-treated B-CMV1A2 cells under any experimental protocol using three different methods for ladder detection (data not shown).

Activation of apoptotic executioner protease caspase-3 was then examined in was then examined in B-CMV1A2 cell cultures dosed with AFB$_1$. B-CMV1A2 cells were exposed to 1 μM AFB$_1$, 1 μM staurosporine, or DMSO for either 4 or 9 h. Proteolysed (activated) caspase-3 was detected in B-CMV1A2 cells exposed to either AFB$_1$ or staurosporine for either 4 or 9 h, but was absent from control (DMSO-treated) cells for 9 h (Fig. 7A). To determine if BCMV1A2 cells possess a functional p53, we examined whether caffeine would cause caspase-3 cleavage. In BCMV-1A2 cells, the 24-h IC$_{50}$ for caffeine was 281 μM as determined by the MTT assay (data not shown). Caspase-3 cleavage was not detected in BCMV-1A2 cells dosed with caffeine (150–450 μM) for 24 h (Fig. 7B).

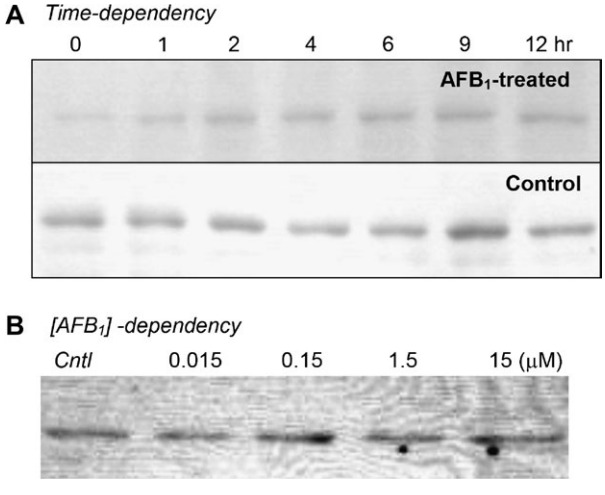

FIG. 4. Representative Western immunoblots showing that the induction of MDM2 expression by AFB$_1$ in B-CMV1A2 over 12 h following 30-min exposures of 1.5 μM AFB$_1$ is time dependent (A), and concentration dependent (6 h after 30-min AFB$_1$ exposures) (B). Cell lysates were prepared as described in Materials and Methods (100,000 cells/20 μl), and 20 μl of the lysate was loaded into gels for analysis. Cell lysate proteins were separated with 10% polyacrylamide SDS–PAGE gels at 125 V for 8 h. Unexposed controls were run at the same time points as a check to rule out changes in protein expression over time (see Fig. 4A bottom frame).

DISCUSSION

In occupations where pulmonary exposures to AFB$_1$-laden grain dusts are common, workers may be at an increased risk of developing lung cancer (Hayes *et al.*, 1984). We previously showed that these CYP-transfected cells activate AFB$_1$ to intermediate(s) that are cytotoxic and form AFB$_1$–DNA adducts. When taking into consideration the expression of

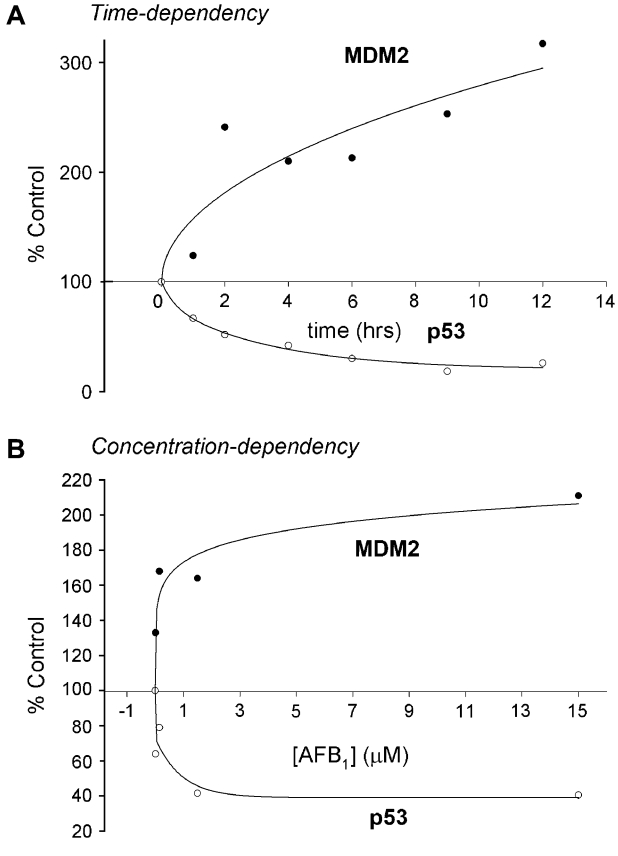

FIG. 5. (A) Plots showing the reflective induction of MDM2 (●) observed with the inhibition of p53 expression (○) in B-CMV1A2 cells over time (0–12 h) after B-CMV1A2 cells are exposed to 1.5 μM AFB$_1$ for 30 min compared to $t = 0$ control. (B) The same trend was detected over a range of AFB$_1$ concentrations (0–15 μM) harvested 6 h after a 30-min exposure. Data points are the mean band densities of $n = 3$ Western blots (from three separate experiments, see Figs. 2–4).

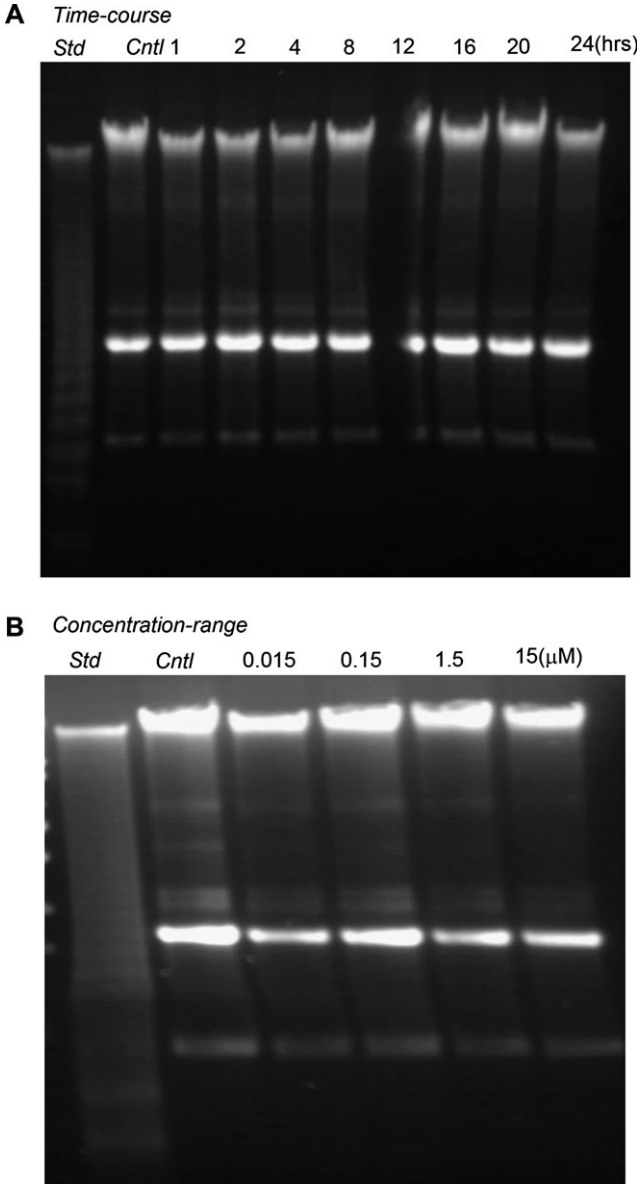

FIG. 6. (A) Representative agarose (2%) gels showing the lack of DNA ladder formation (indicative of apoptosis) in DNA samples from B-CMV1A2 cells after exposure to 1.5 μM AFB_1 for 30 min and harvested at a range of times (0–24 h) after exposure. (B) Representative gels showing the lack of ladder formation in DNA samples from B-CMV1A2 cells 6 h after 30 min exposures to a range of AFB_1 concentrations (0.015–15 μM). Assay was performed as described in Materials and Methods. C = Control, Std = (+) Standard.

CYP mRNA, B-CMV1A2 cells were more efficient at activating AFB_1 at low concentrations (<3 μM), while B3A4 cells were more efficient at higher concentrations (>3 μM) (Van Vleet *et al.*, 2002b). Because of the reported relevance of p53 mutations and altered p53 expression in AFB_1-induced hepatocarcinogenesis (Lee *et al.*, 2000), we sought to determine if AFB_1 exposure has an effect on p53 expression in these human bronchial epithelial cells.

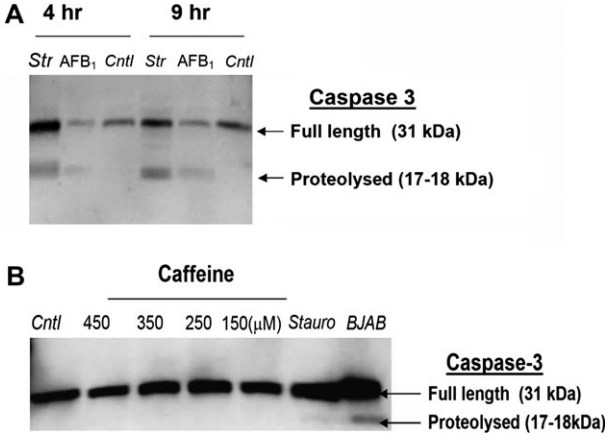

FIG. 7. (A) Representative Western immunoblot showing the proteolytic activation of caspase 3 in B-CMV1A2. Cells were exposed to staurosporine (Str 1 μM; positive control), AFB_1 (1 μM), or DMSO (Cntl 20 μl; negative control) for either 4 or 9 h. B-CMV1A2 cells showed an activation of caspase-3 in staurosporine-and AFB_1-treated cells. DMSO-treated cells showed only full-length (inactive) caspase-3. Cell lysates were prepared as described in Materials and Methods; 0.5 μg of protein was loaded into gels for analysis. Cell lysate proteins were separated with 15% polyacrylamide SDS–PAGE gels at 200 V for 45 min. (B) Western immunoblot of protein from BCMV-1A2 cells dosed with the p53-dependent apoptosis inducer caffeine for 24 h showing a lack of proteolytic activation of caspase-3. Each lane loaded with 24 μg protein, except "Stauro" is 0.012 μg protein of staurosporine-treated BCMV-1A2 cells to demonstrate detection of low levels of cleaved protein, and "BJAB" is 10 μg protein of BJAB-cells treated with MAB631 antagonistic to DR5 as positive controls.

This study indicates that, in contrast to normal cells, the immortalized cells used in this study—BEAS-2B, B3A4, and B-CMV1A2—express p53 constitutively. Since BEAS-2B cells are the progenitor cells of B3A4 and B-CMV1A2, constitutive expression in the latter cell lines was not the result of transfections inducing the expression of CYPs 1A2 and 3A4, but is likely due to SV-40 immortalization. Others have reported similar constitutive p53 expression in BEAS-2B cells (Gerwin *et al.*, 1992). Importantly, our results also indicate that p53 function is impaired by exposure to AFB_1. Our data demonstrate that AFB_1 exposure inhibits p53 expression in B-CMV1A2 and B3A4 cells, an event associated with a reflective increase in MDM2 expression. It has been previously demonstrated that SV-40 affects p53 function. For example, SV-40 large T antigen causes continuous p53 inactivation and leads to immortalization of primary mouse embryonic fibroblasts (Carnero *et al.*, 2000). Other SV-40 immortalized cell lines also constitutively express p53 (Miyazawa *et al.*, 1998; Stein *et al.*, 1991). It was also previously shown that BEAS cells, the progenitor cell line for the B-CMV1A2 and B3A4 cell lines, possess a p53 protein incapable of inducing the expression of downstream proteins under DNA-alkylating conditions (Technau *et al.*, 2001). Researchers from that study noted that p53 expression was frequently decreased in cells exposed to mitomycin C, which is also in agreement with our data showing decreased p53 expression after AFB_1 exposure. An increase in

MDM2 and reduction in p53 was also observed with TCDD in HepG2 cells (Paajarvi *et al*., 2005). Interestingly, in another study, neuroblastoma cells where shown to possess a p53 that was also unable to induce the expression of p21 (and MDM2), after exposure to mitomycin C, even though p53 protein was able to bind DNA (Wolff *et al*., 2001). These discoveries support speculation that SV-40 immortalization not only inactivates p53 from its role in cell cycle control, but also inactivates its ability to protect the integrity of the genome, as seen in some cancer cells. Although these results provide insight into the function of p53 and the effects of SV-40 immortalization on p53 function, they also suggest that BEAS-2B cell physiology may present some limitations to studying the toxicological responses in certain molecular targets.

MDM2 induction, concomitant to the decline in p53 expression, indicates that the decline in p53 expression was not due to cell death from AFB_1 treatment under these conditions. If the decrease were due to a general lack of protein synthesis from cell death, MDM2 would not be induced under these conditions. Other studies have shown that the induction of some MDM2 isoforms can cause a decrease in p53 expression (Carnero *et al*., 2000; Freedman *et al*., 1999), and that MDM2 induction can be independent of functional p53 (Hsing *et al*., 2000). Interestingly, the induction of MDM2 we observed was of the 76-kDa fragment, which typically attenuates the ability of the full-length $p90^{MDM2}$ to decrease the level of p53 thereby increasing p53 (Perry *et al*., 2000).

Further evidence of the lack of p53 function can be seen in the inability of these (BCMV-1A2) SV-40 immortalized lung cells to undergo DNA laddering after treatment with AFB_1 or staurosporine. A lack of functional p53 has been shown to perturb cellular growth arrest and apoptosis (May and May, 1995), while other studies have shown p53-independent induction of apoptosis in some SV-40 immortalized cell types (Gartenhaus *et al*., 1996; Levresse *et al*., 1998). Caffeine has been shown to induce apoptosis in a p53-dependent manner in $p53^{+/+}$ mouse epidermal JB6 C141 cells, resulting in caspase-3 cleavage but not in $p53^{-/-}$ JB6 C141 cells (He *et al*., 2003). We were unable to observe caspase-3 activation in B-CMV1A2 cells by the p53-dependent apoptosis-inducer caffeine, further suggesting that p53 was nonfunctional in these cells. That both AFB_1 and staurosporine were able to activate caspase-3 despite attenuated p53 indicates that apoptosis induced by these two compounds was p53 independent. Apoptosis has been previously reported in BEAS-2B cells (Agopyan *et al*., 2003; Nichols *et al*., 2003), which is consistent with our detection of caspase-3 activation. The absence of DNA ladder in the presence of caspase-3 activation is consistent with previous reports that caspase inhibitors are ineffective at preventing DNA fragmentation during apoptosis in multiple cell types from different species (Villa *et al*., 1998).

The AFB_1-induced inhibition of p53 expression was greater in CYP1A2-expressing than in CYP3A4-expressing cells, except only at the highest AFB_1 (15 μM) concentrations. Earlier studies from our laboratory demonstrated that B-CMV1A2 cells were substantially more efficient at AFB_1 bioactivation to cytotoxic and DNA-alkylating intermediates than were B3A4 cells at low, environmentally relevant concentrations of AFB_1 (Van Vleet *et al*., 2002a,b). In conclusion, the p53-mediated response to AFB_1 treatment may indicate that these cells are at an increased risk of developing mutations. However, in the absence of a typical p53 response, these cells were able to undergo apoptosis, as evidenced by caspase-3 cleavage. The implications of our findings on the usefulness of these cells in *in vitro* studies of the effects of environmental carcinogens such as AFB_1 are unclear. It is possible that the inhalation of AFB_1-contaminated grain dusts may lead to modulation of p53 and cellular death under conditions where appropriate CYPs are expressed in the lung, resulting in adverse health effects.

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