Modulation of benzo[a]pyrene-induced p53 DNA activity by acrolein

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Acrolein, a highly electrophilic \( \alpha,\beta \)-unsaturated aldehyde, is by far the most reactive amongst the aldehydes present in smoke. The relative contribution of acrolein to complex mixture toxicity of smoke at the molecular level remains unknown. The current study examines the ability of acrolein to modulate the effect of benzo[a]pyrene (B[a]P), a major carcinogen found in smoke, on p53. Exposure of human lung adenocarcinoma A549 cells to 1 mM B[a]P for 48 h strongly activated the expression of p53 as seen by western blotting, and its DNA binding as shown by an electrophoretic mobility shift assay. Treatment of A549 cells with a non-lethal dose of acrolein alone (50 fmol/cell for 0.5 h) depleted 80% of total cellular glutathione but had no effect on basal p53 protein levels. When B[a]P-treated cells (48 h) were exposed to acrolein for 0.5 h there was also no effect on B[a]P-induced p53 protein levels. However, acrolein treatments profoundly inhibited the DNA binding of p53 under both basal and B[a]P-induced conditions. Depleting glutathione with buthionine sulfoximine in B[a]P-treated cells to levels similar to those obtained with acrolein decreased p53 DNA binding substantially less than with acrolein. Using a p53 dual luciferase reporter assay, acrolein caused an 83% decrease in the p53 activity induced by B[a]P (1 mM for 24 h post-transfection). The p53 protein that was immunoprecipitated after acrolein treatment was reactive with an anti-acrolein antibody indicating covalent modification. Results from this study suggest that acrolein can inhibit p53 DNA binding and activity by direct covalent modification as well as alteration of intracellular redox status. As both acrolein and B[a]P are found in cigarette smoke, this type of interaction may play an important role in the initiation of lung cancer by altering the tumor suppressor activity of p53.

Introduction

The \( \alpha,\beta \)-unsaturated aldehyde acrolein is a highly electrophilic compound generated as a consequence of combustion and is thus found throughout the environment. Levels in ambient air are estimated at 0.04 to 0.08 p.p.m. Acrolein is also a product of lipid peroxidation (1) meaning this carcinogenic aldehyde (2) is generated endogenously in biological systems. Acrolein’s ubiquitous environmental presence and high reactivity indicates the need for understanding not only how it is toxic itself, but also how it may influence the toxicity of other agents. This is of particular interest in terms of cigarette smoke where acrolein is found at up to 90 p.p.m. for a total emission of up to 230 \( \mu \)g/cigarette (3).

Acrolein’s reactivity is a consequence of the \( \alpha,\beta \)-unsaturated carbon–carbon bond, that reacts via a Michael addition in the presence of a nucleophile to form an alkylated adduct. Cells contain numerous nucleophilic sites, particularly thiols, and the acute toxicity of acrolein at high doses is clearly a result of massive binding to these sites. However, at lower doses our understanding of the pathways that can be affected more selectively by the binding of an electrophile is limited, and only in recent years have the molecular effects of acrolein been investigated (4). Thiolation of the protein-bound acrolein is involved in redox alteration under oxidative stress, whereby oxidative stress generates the increased production of acrolein and its protein adducts in turn potentiate oxidative stress via the depletion of glutathione (GSH) in the cells (2).

Cellular thiol redox status is critical for a variety of biological processes including transcriptional activation of various genes and regulation of cell proliferation, inflammation and apoptosis (5–10). Thiols, particularly GSH, are also critical for cellular antioxidant defences, including protecting lung epithelial cells from oxidant injury and inflammation (8,9). The depletion of these thiols by acrolein would be expected to disrupt a variety of pathways, and the redox sensitive transcription factors nuclear factor-kB (NF-kB) and Activator Protein-1 are inhibited by acrolein (11,12). The mechanism of this inhibition appears to at least in part be due to a direct interaction of acrolein with these proteins.

Mutations in the p53 gene can abrogate its tumor suppressor function leading to lung cancer (13). Components of cigarette smoke can cause such mutations, but other components may block such an action and thus the overall effect of a complex mixture such as cigarette smoke on p53 function is not understood.

Acrolein is a metabolite of the widely used anticancer drug cyclophosphamide (14), and can initiate urinary bladder cancer in rats (15). In addition, administration of acrolein to rats via the portal vein induced putatively pre-neoplastic single cells positive for glutathione S-transferase (16). One recent study showed that acrolein induced the transformation of cells that were pre-treated with B[a]P, but not the untreated cells (17) confirming that an interaction can occur. However, the nature of this interaction has not been studied. Determining whether acrolein has effects on the transcription factor p53 is of interest because: (i) of the redox sensitive nature of this protein; (ii) p53 is one of the most important tumor suppressor factors that prevent transformation; and (iii) p53 is induced in human lung epithelial cells by polycyclic aromatic hydrocarbons such as benzo[a]pyrene (B[a]P) (18). As both acrolein

Abbreviations: B[a]P, benzo[a]pyrene; GSH, glutathione.

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and B[a]P are found in cigarette smoke, any interaction between these agents could have significant functional consequences.

The current study explored the hypothesis that acrolein can alter the activity of p53 protein induced by B[a]P. The results show that exposure of A549 cells (which express wild-type p53) to B[a]P strongly activated the expression, DNA binding and reporter activity of p53. Treatment of these cells with acrolein did not affect B[a]P-induced p53 protein levels. Acrolein did, however, profoundly inhibited the DNA binding and reporter activity of p53 induced by B[a]P. This effect did not appear to simply be a consequence of acrolein-mediated changes in GSH. Anti-acrolein antibodies detected binding of this electrophile to p53 protein indicating that acrolein can alter p53 DNA binding and function, possibly through direct interaction with p53 protein as well as alterations in intracellular redox balance.

Materials and methods

Materials

Acrolein (90%; water and dimers make up the other 10%), L-buthionine SR-sulfoximine (BSO) and B[a]P were obtained from Sigma Chemical Company (St Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Life Technologies (Gaithersburg, MD). The p53 consensus oligonucleotide was purchased from Integrated DNA Technologies (Coraville, IA).

Cell culture and treatments

A549 human lung adenocarcinoma cells, obtained originally from the American Type Culture Collection (Rockville, MD), were cultured in DMEM (pH 7.4) supplemented with 10% (v/v) FBS, 3.7 g/l sodium bicarbonate and 100 mg/ml gentamicin. Cells were maintained at 37°C with 5% CO2. Cultures were passaged at confluency (approximately every 3 days). Cells to be treated were removed from monolayer stock cultures with trypsin-EDTA, counted with a hemacytometer and plated in 6 well dishes (9.6 cm²/well) with a medium volume of 2 ml/well. After incubating at 37°C for 24 h, the cells were treated either with vehicle or 1 μM B[a]P in 2 ml serum free DMEM for 48 h. In some cases, cells were also treated with 5 or 10 mM BSO in filtered DMEM for the final 15 h. Cells were washed with Earls Balanced Salt Solution (Biosource International Inc., Camarillo, CA) and treated for 0.5 h at 37°C with acrolein doses of 50 and 75 fmol/cell. All treatments were performed along with vehicle controls. Following acrolein treatment, the cells were washed with PBS and harvested for nuclear extracts or stored at –80°C for GSH measurements.

Glutathione measurements

Total glutathione (GSH + glutathione disulfide) was measured by HPLC (20) as described previously (11,12). Briefly, cell monolayers were washed twice with PBS, trypsinized, suspended in 1 ml PBS, centrifuged at 2040 g for 4 min and washed once more with PBS. The cellular pellet was lysed in 225 μl 0.02 M EDTA (pH 8.0), sonicated for 3 min and centrifuged to remove the debris. Total protein concentration in the cell lysate was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and compared with a bovine serum albumin standard curve. Total cellular GSH values were calculated using a standard curve derived from GSH (Sigma Chemical). Results are expressed as nmol total GSH/mg protein.

Nuclear extracts

Following the protocol used by Pei et al. (21), pre-washed cells were harvested in 1 ml of PBS, pelleted and washed once more in PBS. Samples were then resuspended in 0.4 ml lysis buffer [1 mM DTT, 0.1 mM EDTA, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.25% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. The pellet was then vortexed and centrifuged at 2040 g for 2 min. The supernatant representing the cytoplasmic extract was discarded. For each sample, 35 μl extract buffer (1 mM DTT, 0.1 mM EDTA, 25% glycerol, 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.5 mM PMSF) was used to resuspend the nuclear pellet. The suspension was rocked for 30 min at 4°C then centrifuged at 2040 g for 4 min. The supernatant collected representing the nuclear extract was analyzed for total protein concentration by the Bio-Rad Protein Assay. The nuclear extracts were stored at –80°C for future use in western blot and EMSA analyses.

Electrophoretic mobility shift assay

Ten micrograms of nuclear extract protein was incubated in a reaction mixture consisting of 25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM DTT and 0.02 A260 units poly d(C–C) for 15 min at 25°C. The p53 oligos (sense 5′-TAC AGA ACA TGT CTA AGC ATG CCG-3′ and antisense) were annealed. DNA binding activity was determined by adding 0.1 ng of p53 oligonucleotide consensus sequence labeled with [γ-32P]ATP (3000–5000 Ci/mmol) to the nuclear extracts and incubating for 15 min at 25°C. Protein–DNA complexes were separated on 4–5% non-denaturing polyacrylamide gels for 2 h at 120 V. Gels were dried and exposed to X-Omat film for autoradiography.

p53 reporter assay

The human p53-luciferase constructs were a gift from Sara Sukumar, Johns Hopkins School of Medicine. The reporter plasmid was generated by excising the 2.4-kb XhoI fragment and the 356-bp XbaI–BamHI fragments of p53 from pClAT and subcloning each into the Smal site of pGL2 basic luciferase reporter vector from Promega (Madison, WI) (22,23). For transfection assays, the cells were seeded at 5 × 104/well in 6 well tissue-culture dishes in DMEM supplemented with 10% fetal bovine serum 24 h before transfection with 2 mg of p53 luc plasmid or its vector using the Panvera TransIT kit (Panvera, Madison, WI). pRL-TK (0.5 mg) was used as an internal control for normalization against Renilla luciferase activity as described before (24). Twenty-four hour post-transfection, the cells were treated with B[a]P for 24 h and then treated with acrolein for 0.5 h. The luciferase activities were assayed after 6 h following acrolein treatment using the Dual Luciferase Assay kit (Promega) and normalized with the Renilla luciferase activity.

Immunoprecipitation

Immunoprecipitation of p53 protein was carried out using the IMMUNOCAT catcher kit (CytoSignal, Irvine, CA). Following treatment, cells (107) were washed with ice-cold PBS, lysed on ice by gentle vortexing with mild lysis buffer containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and incubated on ice for 1 h. Clear supernatant was collected after centrifuging the cell homogenate at 16000 g for 15 min. Lysate (equivalent to 500 μg total protein) was pre-cleared using pre-immune serum tagged protein A/G resin for 2 h at 4°C. Pre-cleared lysate was subjected to immunoprecipitation for p53 protein using 10 μl a monoclonal p53 antibody (Oncogene Research Products, San Diego, CA; Ab-6) overnight at 4°C. The lysate was then incubated with 10 μl protein A/G resin for 30 min at room temperature with gentle shaking. The entire suspension was transferred to a spin filter, pre-coated with mild lysis buffer and the filter was centrifuged at 16000 g for 1 min. After washing the resin twice with mild lysis buffer, it was incubated with 40 μl SDS–PAGE loading buffer for 20 min at room temperature and then centrifuged at 16000 g for 1 min to collect eluted antigen. The eluent was run on 12% SDS–PAGE gels.

Western blotting

Western blotting was done as described previously (12). Protein was transferred on PVDF membranes and blocked with non-fat dry milk for 1 h. The membrane was then incubated overnight with the p53 antibodies (Oncogene Research Products; Ab-6 for p53 and polyclonal Ab-3 for phospho-p53 directed against serine 15), or the antibody that specifically recognizes acrolein–lysine adduct (25) (1:5000 dilution) in 3% milk (Sicto, Tokyo, Japan) for 1 h at room temperature. After membrane washing, horseradish peroxidase conjugated anti-mouse secondary antibodies were used (1:3000 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA). Bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham (Arlington Heights, IL). Band intensities were determined using UN-SCAN-IT software (Silk Scientific, Orem, UT) on scanned images. When appropriate after visualizing the acrolein–lysine adduct membranes were stripped, blocked and re-probed with anti-p53 and compared with anti-acrolein band placement to show acrolein–p53 adducts.

Statistics

Data are expressed as means ± SE. Comparisons between groups were done with a one-way analysis of variance followed by Student Newman–Kuel’s test. A P value of <0.05 was considered to be significant. The EMSA, reporter assay and western blotting for p53 were done three independent times.

Results

Effect of acrolein on B[a]P-mediated increase in p53 and intracellular GSH

As reported by others (21), treatment of A549 cells with B[a]P increases p53 protein levels (Figure 1A, lane 1 versus lane 2). Whereas treatment with 75 fmol/cell acrolein alone for 0.5 h
slightly decreased the basal level of p53, neither 50 nor 75 fmol/cell affected the increased level seen following B[a]P (Figure 1A). Identical results were seen when p53 protein levels were determined 1, 2 and 4 h after acrolein treatment (data not shown). Consistent with our previous report (12), 50 and 75 fmol/cell of acrolein drastically depleted intracellular GSH to ~10% of control levels. Although B[a]P alone increased GSH levels ~2-fold relative to vehicle-treated control cells, acrolein again decreased levels to 10% of control, and ~5% of B[a]P alone (Figure 1B).

**Modulation of B[a]P-induced p53 protein activity**

The B[a]P-mediated increase in p53 protein was accompanied by an increase in DNA binding to the consensus sequence relative to vehicle control (Figure 2A and B). Acrolein treatments (50 and 75 fmol/cell; 0.5 h) decreased both basal and B[a]P-increased p53 DNA binding to levels far below the vehicle-treated control (Figure 2A). This decrease in binding corresponded to an 80% decrease in p53 activity with 50 fmol/cell acrolein relative to B[a]P as shown by a p53 luciferase reporter assay (Figure 2B).

**Effect of GSH depletion by BSO on DNA binding activity**

The mechanism by which acrolein affected p53 binding was studied. As GSH can affect p53 binding and is depleted following acrolein, cells were treated with BSO for the last 15 h of the B[a]P treatment to mimic the effect of acrolein. Five and 10 mM of BSO alone decreased cellular GSH to below 10% of untreated control. A similar extent of depletion was evident when BSO was administered with B[a]P (Figure 3A). There was a 40 and 65% reduction in DNA binding relative to B[a]P alone as a result of depletion of GSH by 5 and 10 mM of BSO, respectively (Figure 3B, lanes 5 and 6 versus lane 2). This was not as great as the reduction seen with acrolein (Figure 2A) despite comparable changes in GSH suggesting additional mechanisms were involved.

**Effect of acrolein on the phosphorylation of p53 protein**

The phosphorylation status of p53 plays an important role in the activity of this protein. Thus, the ability of acrolein to modulate B[a]P-induced p53 activity by altering the
phosphorylation of this protein was investigated. Immunoblotting results using an anti-phospho Ser15p53 antibody indicated that the changes in phospho p53 seen in response to B[a]P paralleled those in non-phosphorylated p53 and were unaffected by acrolein (Figure 4), indicating a lack of effect of acrolein on p53 phosphorylation.

Detection of acrolein adducts on p53 protein

As a reactive compound, acrolein will covalently modify nucleophilic sites on proteins. A monoclonal antibody to the acrolein–lysine adduct detected acrolein adducts on casein incubated with acrolein, but not with native casein demonstrating its specificity (Figure 5A). Immunoprecipitated p53 protein from both control and acrolein-treated A549 cells contained acrolein–lysine adducts (Figure 5B). There was, however, substantially more adducts after acrolein treatment. The finding of acrolein adducts in proteins from untreated cells is not uncommon, perhaps reflecting ongoing endogenous oxidation reactions. In fact, bovine serum albumin contains substantial quantities (data not shown) that may be derived from the peroxidation of bound fatty acids. This is why casein was used as the control protein. Overall, however, the data support the hypothesis that acrolein causes covalent modification of p53 protein.

Discussion

Cellular levels of p53 protein are increased in response to various types of cell stress and DNA damage (26). B[a]P, a potent carcinogen found in tobacco and other smoke, is well-known to damage DNA and to increase p53 protein levels. Under conditions whereby cells are exposed to B[a]P alone, an increase in p53 protein is normally accompanied by an increase in DNA binding and downstream transcriptional activity. The induction of p53 either causes apoptosis or...
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helps in arresting the cell cycle, which in turn allows DNA repair. Both of these mechanisms help in fulfilling its role as a tumor suppressor protein. However, it is important to note that B[a]P exposures in the real world will likely involve complex mixtures. Various components in such mixtures could influence both the pathways that stimulate p53 protein synthesis and, perhaps more likely, the steps involved in p53 DNA binding. This binding appears to be regulated in part by phosphorylation (27) and redox effects (5). Of the nine cysteines found in the p53 DNA binding domain, four are reported to be essential for binding (28). The conformation of p53 appears to be altered following thiol oxidation thereby preventing its binding to specific DNA targets. Effects that disrupt the nature of these cysteines (i.e., oxidation or alklylation) could alter the ability of increased p53 protein to exert any transcriptional effects.

It is of interest that p53 activity is stimulated by Ref-1 (29), a factor maintained in its reduced state by thioredoxin. The importance of thioredoxin reductase in maintaining p53 activity (30) further supports the role of this thiol system in modu-

lating p53. Because acrolein can react with and deplete thioredoxin (unpublished data), and a recent report demonstrates that electrophilic prostaglandins, and other agents with an α,β-unsaturated carbonyl, can inhibit p53 through effects on thioredoxin reductase (31), it as apparent that a myriad of pathways can affect this, and other, redox regulated transcription factors.

The ability of B[a]P to activate p53 in A549 cells has been reported (18,21). This effect appears to be mediated through the induction of NF-κB activity (21). Our previous work showing that acrolein can inhibit NF-κB activation (11) suggested that the combined presence of B[a]P and acrolein in cigarette smoke might alter p53 activation. Although NF-κB activation was not assessed in the current study in response to B[a]P, it is of interest to note that acrolein was unable to alter B[a]P-induced changes in p53 protein, only p53 DNA binding and resultant reporter activity. This strongly suggested that acrolein was acting directly on p53 protein rather than on up- or downstream events. This conclusion was supported by the immunoprecipitation data and acrolein antibody study that showed the existence of acrolein–p53 adducts in A549 cells treated with acrolein.

N-Acetylcysteine has been reported to cause apoptosis in transformed cells by increasing p53 activity post-transcriptionally (32,33). The reduced state of the crucial cysteine residue in the DNA binding site has been speculated to play some role in the p53 activity. Inhibiting the B[a]P-mediated increases in p53 binding as a result of the depletion of GSH by BSO confirms that redox alterations due to thiol imbalance by acrolein may play a role in the decreased binding. However, the GSH effect does not appear to be sufficient to explain the entire decrease in p53 binding activity caused by acrolein, and covalent modification is likely to also play a role. Overall, it is apparent that the ultimate effect of a complex mixture on p53 is dependent on numerous interactions, and that studies looking at individual components do little to discern the ultimate outcome.

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