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Role of human CYP1A1 and NAT2 in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced mutagenicity and DNA adducts

J. BENDALY, K. J. METRY, M. A. DOLL, G. JIANG, J. C. STATES, N. B. SMITH, J. R. NEALE, J. L. HOLLOMAN, W. M. PIERCE, Jr, and D. W. HEIN

Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, and Center for Environmental Genomics and Integrative Biology, University of Louisville School of Medicine, Louisville, KY, USA

Abstract

1. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is carcinogenic in multiple organs and numerous species. Bioactivation of PhIP is initiated by PhIP N²-hydroxylation catalyzed by cytochrome P450s. Following *N*-hydroxylation, *O*-acetylation catalyzed by *N*-acetyltransferase 2 (NAT2) is considered a further possible activation pathway. Genetic polymorphisms in NAT2 may modify cancer risk following exposure.
2. Nucleotide excision repair-deficient Chinese hamster ovary (CHO) cells stably transfected with human cytochrome P4501A1 (*CYP1A1*) and a single copy of either *NAT2*4* (rapid acetylator) or *NAT2*5B* (slow acetylator) alleles were used to test the effect of CYP1A1 and NAT2 polymorphism on PhIP genotoxicity.
3. Cells transfected with *NAT2*4* had significantly higher levels of *N*-hydroxy-PhIP *O*-acetyltransferase ($P = 0.0150$) activity than cells transfected with *NAT2*5B*. Following PhIP treatment, CHO cell lines transfected with *CYP1A1*, *CYP1A1/NAT2*4* and *CYP1A1/NAT2*5B* each showed concentration-dependent cytotoxicity and *hypoxanthine phosphoribosyl transferase* (*hprt*) mutagenesis not observed in untransfected CHO cells.
4. dG-C8-PhIP was the primary DNA adduct formed and levels were dose-dependent in transfected CHO cells in the order: *CYP1A1* < *CYP1A1* & *NAT2*5B* < *CYP1A1* & *NAT2*4* although levels did not differ significantly ($P > 0.05$) following one-way analysis of variance.
5. These results strongly support activation of PhIP by CYP1A1 with little effect of human NAT2 genetic polymorphism on mutagenesis and DNA damage.

Keywords

PhIP; 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; CYP1A1; cytochrome P4501A1; NAT2; *N*-acetyltransferase 2

Introduction

2-Amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) is the most abundant heterocyclic amine mutagen formed during high temperature cooking of meats, accounting for ~70% mean dietary intake of heterocyclic amines in the United States (Keating and Bogan, 2004). PhIP has been identified in smoke condensates from the frying of meat (Thiebaud et al., 1995) and has been detected in airborne particles, diesel-exhaust particulates and incineration ash from garbage-burning plants (Manabe et al., 1993). PhIP is found in cigarette smoke (Manabe et al., 1991) and is a urinary mutagen in smokers of black tobacco (Peluso et al., 1991). PhIP causes mammary, prostate, and colon tumors in the rat and is classified as a probable human carcinogen (Sinha et al., 2000; National Toxicology Program, 2005).

PhIP-induced DNA adduct formation and mutagenesis require metabolic activation, and one important pathway includes *N*-hydroxylation catalyzed by cytochrome P450s (Turesky 2004; 2007; Kim and Guengerich, 2005). Although many arylamine carcinogens undergo *N*-hydroxylation by cytochrome P4501A2, this isoform is primarily restricted to the liver and PhIP carcinogenesis is independent of this P450 in mice (Kimura et al., 2003). Arylamine carcinogens also are activated by cytochrome P4501A1 (CYP1A1) (Murray et al., 1993; Edwards et al., 1994; Hammons et al., 1997; Yamazaki et al., 2004; Kim and Guengerich, 2005; Turesky, 2007) and cDNA-expressed human CYP1A1 exhibited over 15-fold higher affinity for PhIP than human CYP1A2 (Crofts et al., 1998). Since PhIP induces tumors in extrahepatic organs and has relatively high affinity for CYP1A1, it is important to evaluate the role of CYP1A1 in PhIP-induced genotoxicity. *N*-hydroxy-PhIP can be further *O*-acetylated by *N*-acetyltransferase 2 (NAT2) to the acetoxy-derivatives that are highly unstable, leading to electrophilic intermediates that are mutagenic and form DNA adducts (Schut and Snyderwine, 1999).

PhIP-induced DNA adducts primarily form at the C8 position of deoxyguanosine (dG-C8) and can be removed by nucleotide excision repair (NER) (Schut and Snyderwine, 1999). Because DNA adduct levels are a function of environmental exposure and polymorphism in genes involved in carcinogen metabolism, DNA adducts are an informative biomarker for investigations of genetic variation in carcinogen metabolism.

Since heterocyclic amines require metabolic activation to exert their carcinogenic effects, genetic polymorphisms in the enzymes catalyzing the activation and/or detoxification pathways of carcinogen metabolism may account for differences in susceptibility to carcinogens between individuals (Turesky, 2004). Humans exhibit genetic polymorphism in NAT2 resulting in rapid and slow acetylator phenotypes. Relative to the *NAT2**4 reference allele, *NAT2**5B possesses three polymorphisms in the *NAT2* open reading frame: T341C (I114T), C481T (synonymous) and A803G (K268R) and is associated with slow acetylator phenotype (Zang et al., 2004). Epidemiologic studies suggest a role for NAT2 genetic polymorphism in susceptibility to various cancers, but laboratory-based experiments are valuable for investigating metabolism of carcinogens that may contribute to these findings.

O-acetylation of *N*-hydroxy-PhIP has been shown to generate DNA adducts (Snyderwine et al., 2002) suggesting that PhIP-induced DNA damage and subsequent mutagenesis would be greater in rapid than slow NAT2 acetylators. On the other hand, several previous studies have shown that metabolic activation of *N*-hydroxy-PhIP did not require *O*-acetylation (Buonarati and Felton, 1990; Wild et al., 1995; Wu et al., 1997). Thus, we investigated the effects of human CYP1A1 and NAT2 genetic polymorphism on PhIP-induced DNA adducts and mutagenesis in nucleotide excision repair-deficient Chinese hamster ovary (CHO) cells transfected with human *CYP1A1*, human *NAT2**4 (rapid acetylator allele) or human *NAT2**5B (slow acetylator allele).

Materials and Methods

Cell culture

The UV5-CHO cell line, a nucleotide excision repair-deficient derivative of the AA8 line, was obtained from the ATCC (Catalog number: CRL-1865). UV5-CHO is hypersensitive to bulky adduct mutagens and belongs to the excision repair cross complementation group 2. All cells were grown in alpha-modified minimal essential medium (Cambrex) without L-glutamine, ribosides, and deoxyribosides supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 100 units/ml streptomycin (Cambrex), and 2 mM L-glutamine (Cambrex) at 37 °C in 5% CO₂. Media were supplemented with appropriate selective agents to maintain stable transfectants. Construction of UV5/CHO cells expressing human *CYP1A1* and *NAT2*4* or *NAT2*5B* was recently reported and characterized (Bendaly et al., 2007). Briefly, the pFRT/*lacZeo* plasmid (Invitrogen) was transfected into nucleotide excision repair-deficient UV5 cell lines to generate a UV5 cell line containing a single integrated FRT site (UV5FRT). Purified human NADPH cytochrome P450 reductase (POR) and CYP1A1 polymerase chain reaction (PCR) products were digested and ligated into similarly treated pIRES vector and transformed into DH5 α competent cells. The pIRES plasmid containing cDNAs of human *CYP1A1* and *POR* was transfected into the newly established UV5FRT cell line. Those UV5 cells expressing a single FRT site, *CYP1A1*, and *POR* were expanded, and intact geneticin-resistant cells assayed for CYP1A1 activity by measuring 7-ethoxyresorufin *O*-deethylase activity as described previously (Metry et al., 2007). Cell lines with similar levels of 7-ethoxyresorufin *O*-deethylase activity were selected for additional transfection with either *NAT2*4* or *NAT2*5B*.

The open reading frames of *NAT2*4* and *NAT2*5B* were amplified by PCR, digested with *NheI* and *XhoI* (New England Biolabs), and inserted into the similarly prepared pcDNA5/FRT vector (Invitrogen). The pcDNA5/FRT plasmid containing human *NAT2*4* or *NAT2*5B* was co-transfected with pOG44, a Flp recombinase expression plasmid, into UV5FRT cells. Integration of the pcDNA5/FRT construct into the FRT site was confirmed by PCR (Metry et al., 2007). The *NAT2*4*- and *NAT2*5B*-transfected cells were characterized for *N*-acetylation of sulfamethazine, a NAT2-selective substrate (Bendaly et al., 2007).

O-Acetyltransferase assays

N-hydroxy-PhIP *O*-acetyltransferase assays were determined by high performance liquid chromatography (HPLC) as previously described (Metry et al., 2007). Briefly, reaction mixtures containing equal amounts of cell lysate protein, 1 mg/ml deoxyguanosine, 100 μ M *N*-hydroxy-PhIP (Toronto Research Chemicals), and 1 mM acetyl-coenzyme A were incubated at 37°C for 10 min and stopped by the addition of water-saturated ethyl acetate. The reactions were centrifuged for 10 min and the organic phase was transferred, evaporated to dryness, and resuspended in 100 μ l of 10% acetonitrile. HPLC separation was achieved using a gradient of 85:15 sodium perchlorate (pH 2.5)/acetonitrile to 0:100 sodium perchlorate (pH 2.5)/acetonitrile over 10 min. Baseline measurements using extracts of UV5 and UV5/CYP1A1 cells were subtracted from measurements in the *NAT2*4*- and *NAT2*5B*-transfected CHO cell lines.

Cytotoxicity and mutagenesis

Assays for cytotoxicity and mutagenesis were carried out as described previously (Metry et al., 2007). Briefly, cells were grown for 12 doublings, with selective agents in complete hypoxanthine-aminopterin-thymidine medium (30 μ M hypoxanthine, 0.1 μ M aminopterin, and 30 μ M thymidine). Cells were plated at a density of 5×10^5 cells/T-25 flask and incubated for 24 h, after which media were changed and the cells were treated separately for 48 h with various concentrations of PhIP (Toronto Research Chemicals) or vehicle control (0.5% dimethyl

sulfoxide). Survival was determined by colony-forming assay and expressed as percent of vehicle control. The remaining cells were replated and subcultured. After 7 days of growth, cultures were plated for cloning efficiency in complete media and for mutations in complete media containing 40 μ M 6-thioguanine (Sigma). Dishes were seeded with 1×10^5 cells/100 mm dish (10 replicates) and incubated for 7 days; cloning efficiency dishes were seeded with 100 cells/well/six-well plate in triplicate and incubated for 6 days.

Identification and quantitation of PhIP- DNA adducts

Cells grown in 15- cm plates were treated separately with PhIP as described above for the cytotoxicity and mutagenesis assays. Cells were harvested after 48 h of treatment and DNA was extracted and quantified as previously described (Metry et al., 2007). dG-C8-PhIP and dG-C8-PhIP-D3 adduct standards (>95% purity) were obtained from Toronto Research Chemicals. One-tenth volumes each of proteinase K solution (20 mg/mL) and 10% SDS were added to the cell lysate, and the mixture was incubated at 37°C for 60 min. One volume of phenol, equilibrated with 10 mM Tris HCl (pH 8.0), was added to the mixture, which was then vortexed and centrifuged at 3,600 x g for 15 min. The aqueous layer was removed and added to 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) saturated with 10 mM Tris HCl (pH 8.0), which was vortexed and centrifuged. The aqueous layer was removed and added to 1 volume of cold (-20°C) isopropanol, and the mixture was vortexed and centrifuged. The DNA pellet was washed with 70% ethanol and redissolved in 5 mM Tris HCl (pH 7.4) containing 1 mM CaCl_2 , 1 mM ZnCl_2 , and 10 mM MgCl_2 . DNA was quantified by UV spectroscopy using $A_{260 \text{ nm}}$. DNA quality was monitored by UV spectroscopy using $A_{260/280 \text{ nm}}$ and this ratio was consistently above 1.9. DNA samples (200 μ g) added to 1 ng (3.3 adducts per 10^6 DNA bases) deuterated internal standard (dG-C8-PhIP-D3) were digested at 37°C with 10 units DNase I (US Biological) for 1 h followed by 5 units micrococcal nuclease (Sigma), 5 units nuclease P1 (US Biological), 0.01 units spleen phosphodiesterase (Sigma), and 0.01 units snake venom phosphodiesterase (Sigma) for 6 h followed by 5 units alkaline phosphatase (Sigma) overnight. Two volumes of acetonitrile were added to the digest, which was then filtered and concentrated to 100 μ l in a speed vacuum.

Samples were subjected to binary gradient HPLC and introduced into a Micromass Quattro LC triple quadrupole mass spectrometer using a custom-built nanospray as described previously (Metry et al., 2007; Neale et al., 2008). Samples were loaded onto a Inertsil C18 precolumn (5 mm $\times$ 300 μ m i.d., 5 μ m; LC Packings) using Perkin Elmer ABI 140D syringe pumps and a Hewlett Packard 1100 Series autosampler. Multiple reaction monitoring (dwell time, 0.5 s; span, 0.4 Da) was used to measure the $[\text{M}+\text{H}]^+$ to $[(\text{M}-116) + \text{H}]^+$ (loss of deoxyribose) mass transition. A Quattro LC micromass triple quadrupole mass spectrometer equipped with a nanoelectrospray ion source was used for PhIP-DNA adduct quantitation. Multiple reaction monitoring in the electrospray ionization-positive ion mode was carried out using argon as the collision gas. Capillary and cone voltages and collision energies were optimized for cleavage of the glycosidic bond. The dG-C8-PhIP adduct was monitored using the transition from m/z 490 to m/z 374 and the deuterated internal standard (dG-C8-PhIP-D3) was monitored using the transition from m/z 493 to m/z 377.

UvrABC in vitro assay

UvrABC endonuclease was cloned from UvrA^{Bca}, UvrB^{Bca}, and UvrC^{Tma}, and those individual recombinant protein subunits were overexpressed in and purified from E.coli (Jiang et al., 2003; 2004). Plasmid pTHQB04 (5.45 kbp, which contains a 1.5 kbp fragment of the human β -globin gene) was propagated in XL1-Blue (Quan and States, 1996). Form I plasmid DNA was purified by CsCl-ethidium bromide sedimentation equilibrium ultracentrifugation. DNA concentrations were determined by measuring the A_{260} . DNA quality was checked by

agarose gel electrophoresis as well as measurement of the A_{260}/A_{280} ratios (which were 1.8-2.0).

N-hydroxy-PhIP-damaged DNA was prepared with 2 μ g of pTHQB04 plasmid DNA, 2.5 mM acetyl coenzyme A and varying concentrations of *N*-hydroxy-PhIP. Water was substituted for acetyl coenzyme A in controls. The total reaction was incubated at 37°C for 5 min, and stopped with an equal volume of ethyl acetate. The aqueous layer was extracted from the ethyl acetate mixture 4 times. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH = 5.6), and 2.2 volume of cold ethanol. DNA concentrations were determined by measuring the A_{260} . DNA quality was checked by agarose gel electrophoresis as well as measurement of the A_{260}/A_{280} ratios (which were 1.8-2.0).

The plasmid relaxation assay was adapted from that described previously (Jiang et al., 2004). *N*-hydroxy-PhIP-treated plasmid (20 fmol, equivalent to 71 ng pTHQB04, 1 nM of final DNA substrate) was preincubated with UvrA and UvrB in 20 μ l of UvrABC buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 5 mM dithiothreitol, and 1 mM ATP) at 55°C for 30 min, UvrC^{Tma} was added and incubated at 55°C for additional 30 min. Reactions without UvrABC were performed as a no incision control. Reactions were terminated by adding 2 μ l of stop buffer (1% SDS and 200 mM EDTA-Na₂). Cleavage of damaged plasmid-DNA was monitored by following the conversion of Form I plasmid to Form II plasmid. A 20 ng sample of the UvrABC^{Tma} incised DNA was resolved by 1% agarose gel electrophoresis and visualized by staining with SYBR-Gold (Molecular Probes, Eugene Oregon, USA). Fluorescence of resolved bands was quantitated using fluorescence detection mode (Blue excitation, 537 nm) of a Molecular Dynamics Storm 860 Phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

N-hydroxy-PhIP O-acetyltransferase activities

Cell lysates from UV5 and each of the transfected CHO cell lines were tested for their capacity to activate *N*-hydroxy-PhIP to form dG-C8-PhIP adducts. *N*-hydroxy-PhIP *O*-acetyltransferase activity in *CYP1A1/NAT2*4*-transfected cell line was 2.5-fold higher ($P=0.0150$) than in the *CYP1A1/NAT2*5B*-transfected cell line (Fig. 1). Low but detectable levels of *N*-hydroxy-PhIP activation were detected in the UV5 and the *CYP1A1*-transfected cell lines that were subtracted from the experimental measurements in the *CYP1A1/NAT2*-transfected cells.

PhIP-induced cytotoxicity and mutagenesis

The *CYP1A1*-, *CYP1A1/NAT2*4*- and *CYP1A1/NAT2*5B*-transfected CHO cell lines each showed concentration-dependent cytotoxicity (Fig. 2) and *hypoxanthine phosphoribosyl transferase* mutagenesis (Fig. 3) following PhIP treatment, whereas they were not observed in untransfected CHO cells.

PhIP-induced DNA adduct formation

The dG-C8-PhIP standards were characterized by HPLC-tandem mass spectrometry and used to verify the identity of DNA adducts formed in vitro. They were quantitated relative to dG-C8-PhIP-D3 internal standard. One principal PhIP adduct (dG-C8-PhIP), corresponding to m/z 490, was identified in CHO cells incubated with PhIP (Fig. 4). Following PhIP treatment, dG-C8-PhIP DNA adduct levels were dose dependent in cells transfected with *CYP1A1* while the further addition of *NAT2*4* or *NAT2*5B* did not have an appreciable effect (Fig 5). Adduct levels were undetectable in untransfected cells. The observation that cells transfected only with *CYP1A1* were able to bioactivate PhIP raised the question of whether *N*-hydroxy-PhIP required further metabolism by phase II enzymes to generate the DNA reactive electrophile. In order

to investigate whether *N*-hydroxy-PhIP could be activated by acetyl coenzyme A to form DNA adducts without catalysis by NAT2, we incubated *N*-hydroxy-PhIP and acetyl coenzyme A but without NAT2 in the presence of supercoiled plasmid DNA. We assayed for the formation of DNA adducts by UvrABC incision in a plasmid relaxation assay as outlined in Methods. UvrABC is a prokaryotic endonuclease that recognizes and excises bulky DNA adducts such as dG-C8-PhIP. DNA adduct formation, detected by UvrABC incision, was observed in the presence of acetyl coenzyme A despite the absence of catalysis by NAT2 (Fig 6).

Discussion

CYP1A1 and *NAT2* genotypes may increase risk of colorectal cancer from heterocyclic amines (Murtaugh et al., 2005). Recent studies have shown that lung PhIP-DNA adducts were significantly lower in lung tissue of *CYP1A1* null mice but not cytochrome P4501A2 null mice (Ma et al., 2007). Our study tested the effect of human *CYP1A1* in combination with rapid or slow acetylator *NAT2* on mutagenesis and DNA adducts following PhIP exposure. Our study showed that PhIP induced cytotoxicity and mutagenesis in all *CYP1A1*-transfected cell lines but not in the untransfected UV5 cell line. Our plasmid relaxation studies suggest that enzymatic catalysis of *N*-hydroxy-PhIP is not required to generate a DNA reactive electrophile. We observed induction of UvrABC sensitive sites in plasmid DNA incubated with *N*-hydroxy-PhIP in the presence of AcCoA concentrations as low as 20 μ M.

Heterocyclic amines can also be detoxified through glucuronidation and glutathione-S-transferase or via aromatic ring hydroxylation. Once the *N*-hydroxy-metabolite is formed, it can be reduced to the initial carcinogen, deactivated by glutathione-S-transferases or glucuronosyltransferases, or further activated by *N*-acetyltransferases and sulfotransferases. Although we observed differences in *N*-hydroxy-PhIP *O*-acetyltransferase catalytic activity between the *CYP1A1/NAT2*4*- and *CYP1A1/NAT2*5B*- transfected CHO cell lines, *NAT2* did not affect PhIP-induced mutagenesis significantly, consistent with results previously reported in *cytochrome P4501A2/NAT2*-transfected CHO cell lines (Mettry et al., 2007). Although the *NAT2*- effect was slight and not significant ($p > 0.05$), *N*-hydroxy-PhIP-induced mutagenesis and DNA adduct levels were slightly higher in the *CYP1A1/NAT2*4*- than the *CYP1A1/NAT2*5B*-transfected CHO cell line. Previous studies have reported that PhIP DNA adduct levels in human breast tissue are higher in rapid than slow *NAT2* acetylators (Zhu et al., 2003) and have suggested that rapid acetylator *NAT2* phenotype increases colorectal (Lang et al., 1994; Chen et al., 1998; Le Marchand et al., 2001; Lilla et al., 2006; Ognjanovic et al., 2006; Cotterchio et al., 2008), breast (Deitz et al., 2000; Gallicchio et al., 2006), and lung (Chiou et al., 2005) cancer risk in individuals exposed to heterocyclic amine carcinogens. In contrast, other studies carried out with colorectal (Barrett et al., 2003; Barlak et al., 2006), breast (van der Hel et al., 2004; Ochs-Balcome et al., 2007; Mignone et al., 2009), and lung (Barlak et al., 2006) cancer do not support the association with rapid acetylator *NAT2* phenotype. A much more robust effect of *NAT2* phenotype has been noted for mutagenicity and DNA adduct formation from 2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx) (Bendaly et al., 2007). Thus, the effects of *NAT2* phenotype in the gene-environmental studies may reflect heterocyclic amines such as MeIQx more than they do PhIP as noted for colorectal cancer (Ishibe et al., 2002). In summary, these results strongly support activation of PhIP by *CYP1A1* with little effect of human *NAT2* genetic polymorphism on mutagenesis and DNA damage.

Acknowledgments

Declaration of interest

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Figure 1. *N*-hydroxy-PhIP *O*-acetyltransferase activities in cell lysates of *CYP1A1/NAT2*4*- and *CYP1A1/NAT2*5B*-transfected CHO cells. Low but detectable levels of *N*-hydroxy-PhIP activation detected in the UV5 and the *CYP1A1*-transfected cell lines were subtracted from the experimental measurements in the *CYP1A1/NAT2*-transfected cells. Each bar represents Mean $\pm$ S.E.M. for three experiments. **N*-hydroxy-PhIP *O*-acetyltransferase activities were significantly ($p=0.0150$) higher in *CYP1A1/NAT2*4*-than *CYP1A1/NAT2*5B*-transfected CHO cells.

Figure 2. PhIP-induced cytotoxicity in UV5 CHO cell lines. Percent survival on the ordinate is plotted versus PhIP treatment concentration on the abscissa. Each data point represents Mean $\pm$ S.E.M. for three experiments.

Figure 3.

PhIP-induced *hprt* mutants in UV5 CHO cell lines. PhIP-induced *hprt* mutants are plotted on the ordinate versus treatment concentration on the abscissa. Each data point represents Mean $\pm$ S.E.M. for three experiments. *PhIP-induced *hprt* mutants were significantly higher ($p < 0.05$) in the *CYP1A1/NAT2*4* cells at the two highest doses.

Figure 4.

Top panel: mass spectrometric data of dG-C8-PhIP (490 m/z) and dG-C8-PhIP-D3 (493 m/z). Middle panel: main daughter fragment of dG-C8-PhIP (top; 374 m/z) and dG-C8-PhIP-D3 (bottom; 377 m/z). Bottom panel: HPLC chromatograms of dG-C8-PhIP (top; 11.67 min) and dG-C8-PhIP-D3 (bottom; 11.69 min).

Figure 5.

PhIP -induced DNA adduct levels in UV5 CHO cell lines. Adduct levels are plotted on the ordinate versus PhIP treatment concentration on the abscissa. Each data point represents Mean $\pm$ S.E.M. for three experiments (the S.E.M. sometimes falls within the symbol).

Figure 6. UvrABC incision of damaged plasmid DNA as a function of *N*-hydroxy-PhIP concentration in the absence (open squares) and presence (closed inverted triangles) of 2.5 mM AcCoA. Data points represent Means $\pm$ S.E.M. from triplicate samples (the S.E.M. sometimes falls within the symbol).