Diminished Carcinogen Detoxification Is a Novel Mechanism for Hypoxia-inducible Factor 1-mediated Genetic Instability*

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The hypoxia-inducible factor 1 (HIF-1) pathway is induced in many tumors and associated with poorer outcome. The hypoxia-responsive transcription factor HIF-1α dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), which is also an important binding partner for the aryl hydrocarbon receptor (AhR). AhR is an important mediator in the metabolic activation and detoxification of carcinogens, such as the environmental pollutant benzo[a]pyrene (BaP). We hypothesized that HIF-1α activation attenuates BaP-induced AhR-mediated gene expression, which may lead to increased genetic instability and malignant progression. Human lung carcinoma cells (A549) were simultaneously stimulated with CoCl₂, which leads to HIF-1α stabilization and varying concentrations of BaP. Both quantitative PCR and immunoblot analysis indicated that induction of the hypoxia response pathway significantly reduced the levels of AhR downstream targets CYP1A1 and CYP1B1 and AhR protein binding to ARNT. We further demonstrate that the BaP-induced hypoxanthine-guanine phosphoribosyltransferase mutation frequency and γ-H2AX foci were markedly amplified when the HIF-1 pathway was induced. BaP-DNA adducts were only marginally increased, and transient strand breaks were diminished by HIF-1 induction, indicating changes in DNA repair. These data indicate that concurrent exposure of tumor cells to hypoxia and exogenous genotoxins can enhance genetic instability.

Hypoxia is a characteristic of a number of pathologies, including cancer (1), inflammation (2), and kidney disease (3). It promotes genetic instability, as exposure of cultured cells to hypoxia results in an elevated mutation frequency (4, 5). Several mechanisms have been proposed for this observation, including down-regulation of DNA repair (6) and induction of DNA damage by reactive oxygen species produced during reoxygenation (7). In many tumors, the hypoxia-responsive transcription factor HIF-1α² is overexpressed (8) and is associated, together with its downstream targets, with poorer outcome (9). We propose a novel alternative mechanism whereby overexpression of the HIF-1 pathway alters the mutagenicity of exogenous carcinogens, leading to a mutagenic phenotype.

HIF-1α is regarded as the primary molecular switch to alter gene expression in response to reduced oxygen tension. Under normoxia, HIF-1α is hydroxylated by an oxygen-dependent prolyl hydroxylase (termed HIF-PH). This modification targets it for ubiquitination and subsequent degradation (10). In contrast, under hypoxic conditions, HIF-1α becomes stabilized and is translocated into the nucleus, where it forms HIF-1, a heterodimer with HIF-1β also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). ARNT is constitutionally expressed irrespective of oxygen tension (11). Subsequently, HIF-1 binds to the (A/G)CGTG consensus sequence in the hypoxia-responsive elements of the promoter/enhancer regions in the DNA (12), where it drives the expression of a wide array of hypoxia-inducible genes, including vascular endothelial growth factor (13), glucose transporter 1 (14), and carbonic anhydrase IX (CA-IX) (15). These genes are crucial in mediating the cellular responses to hypoxia.

Interestingly, in addition to HIF-1α, ARNT also forms a dimer with the aryl hydrocarbon receptor (AhR). AhR is a receptor for environmental pollutants such as dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) and polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene (BaP)). Polycyclic aromatic hydrocarbons are widely distributed environmental contaminants produced as by-products of combustion processes such as in coke ovens, cigarette smoking, and charcoal grilling of food. Upon ligand binding, AhR translocates into the nucleus, dimerizes with ARNT, and activates gene expression by binding to the TNGCGTG consensus sequence in the xenobiotic-responsive elements of target genes (16). This leads to the up-regulation of a multitude of genes, including the cytochrome P450 isoforms CYP1A1 and CYP1B1. These enzymes metabolize their polycyclic aromatic hydrocarbon substrates to more soluble and excretable products but at the same time may activate certain metabolites into highly reactive forms (17). BaP is a classic example and is enzymatically activated to BaP-7,8-dihydridiol-9,10-epoxide (BPDE) by cytochrome P450 anhydrase IX; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BaP, benzo[a]pyrene; BPDE, BaP-7,8-dihydridiol-9,10-epoxide; PBS, phosphate-buffered saline; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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1 The abbreviations used are: HIF-1, hypoxia-inducible factor 1; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; CA-IX, carbonic acid hydrase IX; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BaP, benzo[a]pyrene; BPDE, BaP-7,8-dihydridiol-9,10-epoxide; PBS, phosphate-buffered saline; HPRT, hypoxanthine-guanine phosphoribosyltransferase.
enzymes. BPDE can covalently bind to DNA, forming highly mutagenic DNA adducts (18).

Because HIF-1α/ARNT is a component of the AhR and HIF-1 signaling pathways, it has been suggested that simultaneous activation of the two pathways can result in competition for ARNT, thereby reducing the activity of both the HIF-1- and AhR-mediated response (19). This implies that, in hypoxic regions, the availability of HIF-1α/ARNT for the metabolism of carcinogens is attenuated. Cross-talk between these two pathways has previously been demonstrated, but studies on the exposure of various hepatoma cell lines to hypoxia and AhR ligands (mostly TCDD) revealed inconsistent interactive results. Whereas Chan et al. (20) and Nie et al. (21) demonstrated mutual inhibition between the HIF-1 and AhR pathways, Gradin et al. (19), Gassmann et al. (22), and Pollenz et al. (23) showed that HIF-1 pathway activation inhibits the AhR pathway but not vice versa. All these studies showed some level of cross-talk between the two pathways; however, none demonstrated that the cross-talk had any biological significance, as, for instance, increased genetic instability. The above-mentioned studies used TCDD as an activator of AhR, and although TCDD is the strongest activator of AhR, it is not metabolized by the CYP proteins induced by the AhR pathway. Importantly, in this study, we used BaP, as it induces downstream CYP expression that results in self-metabolism. Thus, we are studying an interaction that is physiological and relevant to carcinogenesis.

The aim of this study was to determine the involvement of HIF-1 induction in detoxification of carcinogens. First, we determined the level of HIF-1 and AhR pathway cross-talk by quantitative PCR, immunoprecipitation, and immunoblotting.

**Experimental Procedures**

**Cell Culture and Treatment**—A549 cells (human epithelial lung carcinoma cells; obtained from the American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Sigma) and maintained at 37 °C in a 5% CO₂ atmosphere. Cells (1 × 10⁶) were treated with or without the HIF-1α-stabilizing agent cobalt chloride (300 μM; Sigma). After 1 h at 37 °C, BaP (Sigma) in Me₂SO was added to the medium to final concentrations of 0–10 μM. Concentrations of Me₂SO did not exceed 0.5%. After a further 18 h, the cells were harvested. For the determination of the effect on adduct formation and mutation frequency, we used previously established A549 cell lines stably expressing ubiquitin Lys⁶⁵ mutants (K63R) (24).

**Real-time Quantitative PCR**—After 18 h of incubation, the medium was removed. Cells were washed twice with phosphate-buffered saline (PBS), and TRIzol (Invitrogen) was added. Total RNA was isolated according to the manufacturer's instructions. The quantity and quality of each RNA sample were measured spectrophotometrically using NanoDrop 1000 (Thermo Scientific, Waltham, MA). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad) starting with 1 μg of RNA. cDNA was diluted 25-fold in RNase-free water. Real-time PCR was performed using the MyiQ single-color RT-PCR detection system (Bio-Rad) using SYBR Green Supermix (Bio-Rad), 5 μl of diluted cDNA, and 0.3 μM primers (Table 1) in a total volume of 25 μl. Samples were amplified under the following conditions: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. PCR was checked for aspecific products by performing a melting curve analysis (65–95 °C). Data were analyzed using the MyiQ software system (Bio-Rad) and were expressed as relative gene expression (ΔΔCt) method. Two stably expressed genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase, were included as references.

**Western Blot Assay**—Following treatment with CoCl₂ and BaP, cells were washed twice, and proteins were extracted in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5) for 30 min on ice. Samples were sonicated, and protein concentrations were determined by the Lowry assay (Bio-Rad) with bovine serum albumin as a standard. Proteins were separated on a 10% SDS-polyacrylamide gel (150 V, 2 h) and transferred (100 V, 1 h) to a nitrocellulose membrane (GE Healthcare). Membranes were blocked for 1 h with 5% bovine serum albumin and subsequently incubated overnight at 4 °C with a 1:2000 dilution of mouse anti-CA-IX monoclonal antibody (Novus Biologicals), a 1:1000 dilution of rabbit anti-Chk1 monoclonal antibody (Cell Signaling, Danvers, MA), or a 1:1000 dilution of mouse anti-Chk1 monoclonal antibody (Abcam, Cambridge, UK). After washing, blots were incubated for 1 h with a 1:5000 dilution of either horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling) or horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized by enhanced chemiluminescence (GE Healthcare). Mouse anti-β-actin monoclonal antibody (Sigma) was used as a loading control.

**Immunoprecipitation**—After CoCl₂ and BaP treatment as described above, cells were washed twice, and proteins were

**Table 1**

| Gene | Sequence (5' → 3') |
|------|-------------------|
| B-Actin Forward primer | CAGGCAACACCAACACAGTAT |
| B-Actin Reverse primer | GCCATCACCACGAGATTTCT |
| GAPDH Forward primer | GCACCAAGAACTTGGACCA |
| GAPDH Reverse primer | TGGCACTGTGGAGCTG |
| CA-IX Forward primer | CATCTTGGCCTTGTCTGGGA |
| CA-IX Reverse primer | GCTCACAAGCTCTTCCTTT |
| CYP1A1 Forward primer | CTTCTTGGAGACCTCCGACACT |
| CYP1A1 Reverse primer | CTTTTACAACTTGTGCTT |
| CYP1B1 Forward primer | AGTCAAGCGAAGATGGA |
| CYP1B1 Reverse primer | GGCATGCTTCTCATAAAG |

**Primer sequences for real-time reverse transcription-PCR**

| Gene | Primer |
|------|--------|
| GAPDH | Primer | Forward primer |
| GAPDH | Reverse primer |
| CA-IX | Primer |
| CA-IX | Reverse primer |
| CYP1A1 | Primer |
| CYP1A1 | Reverse primer |
| CYP1B1 | Primer |
| CYP1B1 | Reverse primer |

**GAPDH, glyceraldehyde-3-phosphate dehydrogenase.**
extracted in lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 7.5) for 30 min on ice. Samples were sonicated; soluble fractions were recovered; proteins were quantified using the Lowry protein assay; and 250 μg of protein was incubated with a 1:50 dilution of rabbit anti-HIF-1β/ARNT antibody (C15A11, Cell Signaling) overnight at 4 °C. The following day, lysates were incubated for 48 h at 4 °C with 100 μl of GammaBind Sepharose beads (GE Healthcare). Beads were washed five times with lysis buffer, and proteins were eluted by boiling in Laemmli SDS sample buffer for 5 min at 95 °C. Western blotting was performed as described above using mouse anti-HIF-1α monoclonal antibody (1:1000; BD Biosciences) or mouse anti-AhR monoclonal antibody 3B12 (1:500; Abnova, Heidelberg, Germany).

**Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) Mutation Analysis**—Cells were cultured in hypoxanthine-, aminopterin-, and thymidine-supplemented culture medium for 1 week to eliminate background mutations. Cells were then seeded at 1 × 10^6 cells/10-cm dish and exposed to CoCl₂ and BaP as described previously. Subsequently, cells were passaged for 1 week to allow for phenotypic expression of the acquired mutations. HPRT mutant cells were selected in medium supplemented with 30 μM 6-thioguanine. After 14–21 days, cells were fixed and stained with 2% bromphenol blue in 70% ethanol. After staining, the colonies were counted and corrected for plating efficiency.

**γ-H2AX Staining**—Cells were seeded on glass cover slides before treatment with CoCl₂ and BaP. After treatment, cells were fixed with 4% formaldehyde at room temperature for 10 min, washed with PBS, permeabilized with 0.2% Triton X-100 for 5 min at 4 °C, washed twice for 5 min with PBS, and blocked in PBS with 1% bovine serum albumin and 0.5% Tween 20 for 1 h at 37 °C. The coverslips were incubated with a 1:1000 dilution of anti-γ-H2AX antibody clone JBW301 (Upstate, Billerica, MA) for 2 h at 37 °C, washed twice with PBS for 5 min, and incubated with a 1:1000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) for 1 h at 37 °C. Cells were washed twice with PBS for 5 min and dehydrated in 70, 90, and 100% ethanol for 3 min each. Cells were stained with 4′,6-diamidino-2-phenylindole and mounted with VECTASHIELD mounting medium (Vector Laboratories, Peterborough, UK). Digital fluorescent images were taken, and damage was assessed. Positive cells were defined as having more than three foci/cell.

**32P Post-labeling of BPDE-DNA Adducts**—After removal of the aqueous phase during RNA isolation using TRIzol, the remaining phases were used for DNA isolation according to the manufacturer’s protocol. DNA adduct levels were determined according to the nuclease P1 enrichment technique originally described by Reddy and Randerath (25) with the modifications described by Godschalk et al. (26). In all experiments, three BPDE-DNA standards with known BPDE-DNA adduct levels (one adduct per 10^6, 10^7, and 10^8 normal nucleotides) were analyzed in parallel for quantification purposes. Adduct spots on the chromatograms were quantified using phosphorimaging technology (Fujifilm FLA-3000).

**Comet Assay**—Following CoCl₂ and BaP treatment, cell suspensions were diluted 1:4 in 0.5% low melting point agarose and added to microscope slides precoated with a layer of 1.5% normal melting point agarose and put at 4 °C for 45 min. Cells were lysed (0.25 M NaOH, 0.1 M EDTA, 0.01 M Tris, 2.5 M NaCl, 1% Triton X-100, and 10% Me₂SO, adjusted to pH 10) for 1 h at 4 °C, washed with PBS, placed in electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 40 min for denaturation, and subsequently separated by electrophoresis for 30 min at 25 V and 300 mA. The slides were washed twice with PBS for 10 min, stained with ethidium bromide (10 μg/ml), and visualized using a Zeiss Axioskop fluorescent microscope. 50 randomly selected nuclei were analyzed per slide using the Comet Assay III software program (Perceptive Instruments, Haverhill, UK).

**Statistical Analysis**—Results are expressed as the means ± S.E., and experiments were repeated up to five times. GraphPad Prism 4 was used for statistical analysis. To examine differences between cells incubated with or without CoCl₂, a two-way analysis of variance test with Bonferroni post-hoc multiple comparison correction was used. To assess differences between different BaP concentrations, a one-way analysis of variance was used. Differences were considered to be statistically significant when p < 0.05.

**RESULTS**

**BaP Down-regulates in a Dose-dependent Manner the CoCl₂-mediated Induction of CA-IX**—The effect of BaP on the HIF-1 pathway was tested by determining changes in mRNA and protein levels of CA-IX. A549 cells were exposed to 300 μM CoCl₂ to stabilize HIF-1α and to various concentrations of BaP (Fig. 1). After exposure to CoCl₂, an ∼17-fold increase in CA-IX mRNA levels was observed compared with cells without CoCl₂. After the addition of BaP, a dose-dependent decrease was detected that reached 36% at 10 μM BaP (p < 0.05). In addition, CA-IX was also statistically significantly down-regulated when incubated with only BaP (p < 0.05). The same conditions were used to test the effect of BaP on the protein levels of CA-IX. An ∼31-fold increase in CA-IX protein levels was observed when cells were exposed to CoCl₂ compared with cells without CoCl₂. After the addition of BaP, a dose-dependent decrease was detected that reached 53% reduction was already observed after incubation with 1 μM BaP (p < 0.05). After treatment with 10 μM BaP, the reduction in CA-IX protein levels was further increased to ∼66% (p < 0.05). In contrast to the mRNA analy-
sis, the influence of BaP alone on the CA-IX protein levels was absent.

HIF-1 Induction Reduces BaP-induced CYP1A1 and CYP1B1 mRNA Expression—To determine the influence of the induction of the HIF-1 pathway on the AhR pathway, the mRNA levels of CYP1A1 and CYP1B1 were investigated in CoCl2-treated cells. As expected, BaP caused a dose-dependent increase in both CYP1A1 (Fig. 3A) and CYP1B1 (Fig. 3B) mRNA levels. This dose-dependent increase in CYP1A1 mRNA levels was down-regulated by incubation with CoCl2. Without CoCl2, cells showed an ∼24–30-fold increase when incubated with 1 and 10 μM BaP, respectively, but upon simultaneous incubation with CoCl2, this induction of mRNA levels was ∼60% lower. CYP1B1 mRNA levels showed the same pattern, although the increase by BaP was less pronounced; cells incubated with BaP alone showed an ∼8- and ∼9-fold increase when incubated with 1 and 10 μM BaP, respectively. When simultaneously incubated with CoCl2, this induction reached only 68 and 56% of the levels without CoCl2 incubation, respectively.

Competition for ARNT Results in a Reduction in HIF-1 and AhR Protein Bound to ARNT—To directly assess the influence of pathway convergence on protein complex interaction, HIF-1 and AhR-bound ARNT was determined by immunoprecipitation. HIF-1 bound to ARNT increased 7-fold after exposure to CoCl2 (Fig. 4). After the addition of BaP, dose-dependent decreases were detected in the CoCl2-treated cells of ∼24 and ∼48% at 0.1 and 1 μM BaP, respectively. AhR bound to ARNT was 40–75% lower when the HIF-1 pathway was induced compared with that in cells without the addition of CoCl2 (Fig. 4).

HIF-1 Induction Increases BaP-induced Mutation Frequency—To further determine whether this HIF-1-mediated attenuation of the AhR pathway results in changes to the mutagenic phenotype, A549 cells stably expressing K63R ubiquitin were exposed to CoCl2 and various concentrations of BaP, and mutations in the HPRT gene were assessed. This cell line has a diminished capacity to perform error-free bypass of DNA lesions, greatly enhancing the carcinogen-associated mutagenicity of BaP without changing its metabolic activation (27). Cells incubated with CoCl2 alone already showed a slight although not yet significant increase in mutation frequency compared with control cells (∼6-fold) (Fig. 5). In addition, the mutation frequency was significantly induced by ∼10- and ∼21-fold when cells were incubated with 0.1 and 1 μM BaP, respectively, without CoCl2. Interestingly, after the combined incubation with CoCl2 and 0.1 and 1 μM BaP, mutation frequencies were further increased by ∼46- and ∼66-fold, respectively, compared with unexposed cells.

HIF-1 Induction Increases BaP-induced γ-H2AX Foci—To further confirm the increased genomic instability, we assessed γ-H2AX focus formation following treatment with CoCl2 and
BaP. The addition of BaP increased the amount of damaged cells to ~46% (from ~24% in untreated and CoCl2 alone-treated cells) (Table 2). With the addition of CoCl2 to the BaP-treated cells, the number of damaged cells further increased to ~59% and ~53% at 0.1 and 1 μM BaP, respectively.

**HIF-1 Induction Increases Chk1 Phosphorylation**—To determine whether the observed increase in γ-H2AX foci was associated with replication fork blockage, Chk1 phosphorylation was measured by immunoblotting. Interestingly, CoCl2 alone was sufficient to induce a 1.6-fold increase in phosphorylation of Chk1 (Fig. 6), whereas BaP alone induced an ~1.5-fold increase compared with no treatment. This BaP effect was further augmented with the addition of CoCl2 to a combined maximum effect of 2.4-fold increase at 0.1 μM BaP.

**HIF-1 Induction May Alter BaP-induced Adduct Formation**—Because the expression of metabolic enzymes for BaP was altered by HIF induction, one possible explanation for the increased mutant frequency upon the combined exposure to BaP and CoCl2 would be an increased formation of reactive derivatives and the subsequent formation of promutagenic BPDE-DNA adducts. Therefore, BPDE-DNA adducts were measured in cells incubated with both CoCl2 and 0.1 or 1 μM BaP. DNA adducts showed a trend to be higher (20–50%) upon HIF-1 induction compared with BaP alone (Fig. 7).

**HIF-1 Induction Decreases DNA Repair-associated Strand Breaks**—One known mechanism that could further explain the increased mutagenicity of combined BaP and CoCl2 treatment would be inhibition of DNA repair processes. DNA repair induces transient strand breaks that can be detected by the comet assay. Indeed, as expected, the number of strand breaks was dose-dependently increased by the addition of BaP, and importantly, induction of the HIF-1 pathway reduced the comet size by 12–25% (Fig. 8).

**DISCUSSION**

Hypoxia has been reported in several studies to increase mutation frequencies (4, 5). Understanding the cause for this mutation induction is important in determining the role of hypoxia in genetic instability. We propose a novel mechanism in which the interaction between induction of the HIF-1 pathway and AhR leads to an increase in chemical-induced mutations, which is of major relevance for the preclinical stages of carcinogenesis; humans are continuously exposed to carcinogens, and hypoxia may already occur in the early phases of disease development. In this study, we have provided evidence that the combined stimulation of AhR by BaP and the HIF-1 pathway leads to an increase in HPRT mutations and thus disrupts genetic stability.

To determine whether AhR stimulation could result in an alteration of HIF-1-mediated signaling, cells were simulta-
neously incubated with BaP to induce the AhR pathway and with CoCl₂ to induce the HIF-1 pathway. Cells under hypoxic conditions have to undergo metabolic adaptations to survive, and the expression of many genes changes as a result. We used CA-IX as an exemplary downstream gene to test the influence of BaP on the HIF-1 pathway because CA-IX is strongly up-regulated by hypoxia (15) and it is a prognostic factor for many cancers (28). Our results show that BaP exposure inhibited both the basal levels and the induction of CA-IX by CoCl₂ (Fig. 1). Although the mechanisms responsible for expression of basal levels of CA-IX regulation have not been studied, this could suggest that CA-IX is partly induced by HIF-1-independent mechanisms. A recently published study by Takacova et al. (29) showed that, next to mRNA levels, the protein levels decreased similarly after exposure to TCDD. To confirm that this effect also occurs with BaP, we measured the CA-IX protein levels, and indeed, a combined exposure to CoCl₂ and BaP led to a statistically significant dose-dependent decrease in protein concentrations. This dose-dependent decrease in protein levels was even stronger compared with the mRNA levels (Fig. 2). In contrast with the mRNA results, BaP alone did not reduce CA-IX protein levels. These results confirm the hypothesis that induction of the AhR pathway leads to downstream effects in the HIF-1 pathway.

To determine whether HIF-1 induction may influence the metabolism of BaP, we studied the effect of HIF-1 activation on CYP1A1 and CYP1B1 gene expression induced by BaP. Consistent with previous reports, we found a decrease in BaP-induced CYP1A1 mRNA levels (Fig. 3A) due to CoCl₂ (20, 30). This CoCl₂-reduced AhR-mediated induction was also observed for CYP1B1 expression, although to a lesser extent (Fig. 3B), which was also observed in a previous study (30). We also confirm that CYP1B1 levels were already reduced by CoCl₂ even without the addition of BaP, whereas CYP1A1 was reduced when simultaneously incubated with BaP (20, 30). This could be explained by the lower base-line expression of CYP1A1 compared with CYP1B1. However, the effect of CoCl₂ on CYP1A1 expression was stronger compared with its effect on CYP1B1 expression, which is biologically relevant because CYP1A1 has a greater capacity for BaP metabolism than does CYP1B1 (31). These results indicate that induction of the HIF-1 pathway leads to downstream effects in the AhR pathway.

To investigate if this mutual interaction is a result of the competition for ARNT, we determined the amount of HIF-1α and AhR protein bound to ARNT and whether this is influenced by co-stimulation with CoCl₂ and BaP. Our results show that ARNT-bound HIF-1α decreased in a dose-dependent manner with increasing BaP concentrations (Fig. 4). Furthermore, AhR bound to ARNT was also reduced upon HIF-1 induction. This confirms our mRNA transcription results, suggesting that fewer complexes are available to bind to xenobiotic- and hypoxia-responsive elements on the DNA, which leads to decreased mRNA and protein levels of CYP1A1, CYP1B1, and CA-IX. Our data are in agreement with those of Chan et al. (20) and Nie et al. (21), who observed that AhR inhibits HIF-1α/ARNT interaction with the DNA and that HIF-1α lowers the amount of AhR-ARNT complexes bound to the DNA. Taken together, our results demonstrate a mutual interaction between the AhR and HIF-1 pathways.

We further investigated whether this interaction leads to an increase in HPRT mutation frequency by using a previously developed highly sensitive A549 mutant cell line (24). We earlier described that the mutants and A549 cells behave in an identical manner with regard to cell growth, clonogenic survival, and BPDE-DNA adduct formation (18, 24). Our present results show that the amount of BaP-induced mutant cells was significantly higher when the HIF-1 pathway was accordingly induced. A more than additive effect was observed because the amount of mutations caused by both CoCl₂ and BaP is more than the sum of CoCl₂ and BaP alone. These results imply that when both pathways are triggered at the same time, the mutagenic effects of chemical carcinogens may be potentiated, resulting in enhanced genetic instability. Additionally, the well-established DNA damage marker γ-H2AX was used to further investigate genetic instability (Table 2). HIF-1 induction itself does not induce more damaged cells. On the other hand, treatment with BaP enhances the amount of damaged cells, which was further increased by the concomitant induction of the HIF-1 pathway. As this induction of γ-H2AX staining may be associated with replication fork blockage (32), we examined Chk1 phosphorylation. Upon stalling of the replication machinery, ATR (ataxia telangiectasia- and Rad3-related protein) is recruited to these sites, where it phosphorylates Chk1 at Ser317 and Ser345 (33). We determined that the BaP-induced phosphorylation of Chk1 was further increased by HIF-1 induction (Fig. 6). Taken together, these experiments additionally confirm the hypothesis that inducing both pathways leads to greater genomic instability.

Because we observed an effect of HIF-1 induction on the expression of BaP-metabolizing enzymes, the increased mutation frequency may be explained by an accumulation of promutagenic DNA adducts (Fig. 7). Interestingly, there was only a small rise in adduct levels due to incubation with CoCl₂. As the bulk of adducts are removed by the nucleotide excision repair pathway, this may mask the significance of remaining lesions entering S phase. This is further supported by the observed increase in γ-H2AX foci and the increased phosphorylation of Chk1, suggesting adduct-associated blockages of replication forks. Alternatively, other mechanisms related to BaP mutagenicity may be involved. For example, it has been reported that the most relevant repair mechanism for BPDE-DNA adducts, nucleotide excision repair, is inhibited by hypoxia (34). Therefore, although the initial adduct levels do not differ substantially, it may be that, upon HIF-1 induction, crucial DNA lesions may be less well removed, leading to higher mutation rates upon proliferation. Still, the relation between BaP adducts and mutagenesis is complex, and multiple factors (both structural and biological) are able to influence the mutagenicity of BaP-related DNA adducts (35).

To further elucidate the mechanism behind the increased mutation frequency, we quantitated DNA strand breaks using the comet assay. BaP does not induce strand breaks directly because damage is induced after metabolism to BPDE and the formation of adducts. During the repair of these adducts, transient single-strand breaks are generated (36). Our data show
that the amount of strand breaks was lower when the HIF-1 pathway was induced compared with control conditions. It is interesting to note that, even without treatment with BaP, the number of breaks was lower under conditions in which HIF-1 was induced compared with normal conditions. The data suggest that the decrease we observed in breaks due to the induction of HIF-1 is not explained by a decrease in DNA damage but rather a decrease in repair incisions. This indicates and confirms that hypoxia reduces nucleotide excision repair capacity (34), which is relevant for removal of bulky polymerase-blocking lesions.

We have shown that alteration of the HIF-1 pathway by CoCl2 results in a mutagenic phenotype. It is interesting to note that a secondary mode of HIF induction has been attributed to either oncogene activation (37, 38) or loss-of-function mutations in tumor suppressor genes (39, 40). This may suggest an important role in malignant progression, as oncogene or tumor suppressor gene alteration may lead to subsequent genetic instability via HIF-1 modulation of carcinogen detoxification.

Interestingly, our results suggest an overall detrimental effect on survival, and one may question the evolutionary benefits of this complex pathway interaction. This further underscores the poorly understood physiological role of AhR, as even its natural ligand is still unknown.

In this study, have we demonstrated that there is a two-way interaction between the HIF-1 and AhR pathways. We have provided evidence that CoCl2 results in an increase in BaP-induced mutation frequency. These data support the idea that the modulation of carcinogen metabolism may be an important mechanism for the observed HIF-1-mediated genetic instability, suggesting a potential role in cancer development.

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