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Loss of VHL in RCC reduces repair and alters cellular response to benzo[a]pyrene

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Mutations of the von Hippel-Lindau (VHL) tumor suppressor gene occur in the majority of sporadic renal-cell carcinomas (RCC). Loss of VHL function is associated with stabilization of hypoxia-inducible factor α (HIFα). We and others demonstrated that there is a two-way interaction between the aryl hydrocarbon receptor, which is an important mediator in the metabolic activation and detoxification of carcinogens, and the HIF1-pathway leading to an increased genetic instability when both pathways are simultaneously activated. The aim of this study was to investigate how environmental carcinogens, such as benzo[a]pyrene (BaP), which can be metabolically activated to BaP-7,8-dihydroxy-9,10-epoxide (BPDE) play a role in the etiology of RCC. We exposed VHL-deficient RCC4 cells, in which HIFα is stabilized regardless of oxygen tension, to 0.1 μM BaP for 18 h. The mutagenic BPDE-DNA adduct levels were increased in HIFα stabilized cells. Using qRT-PCR, we demonstrated that absence of VHL significantly induced the mRNA levels of AhR downstream target CYP1A1. Furthermore, HPLC analysis indicated that loss of VHL increased the concentration of BaP-7,8-dihydroxyflavylid, the pre-cursor metabolite of BPDE. Interestingly, the capacity to repair BPDE-DNA adducts in the HIFα stabilized RCC4 cells, was markedly reduced. Taken together, these data indicate that loss of VHL affects BaP-mediated genotoxic responses in RCC and decreases repair capacity.

Keywords: nucleotide excision repair, metabolism, carcinogens, renal-cell carcinoma, von Hippel-Lindau

INTRODUCTION

Renal-cell carcinoma (RCC) is the most common type of kidney cancer in adults and accounts for 4% of all cancers (1). The most frequently observed genetic alteration in RCC is the somatic mutation of the von Hippel-Lindau (VHL) tumor suppressor gene (2, 3).

The VHL protein (pVHL) is a crucial regulator of the oxygen sensing pathway, which involves the transcription factor hypoxia-inducible factor alpha (HIFα) (2, 4). In the presence of oxygen, HIFα is hydroxylated by an oxygen-dependent prolyl hydroxylase (HIF-PH) (5). An E3 ubiquitin ligase complex containing the pVHL recognizes the hydroxylated HIFα, and targets it for ubiquitination (6) and subsequently proteasomal degradation (7). Under hypoxic conditions, HIFα is not prolyl-hydroxylated and thus unrecognized by pVHL.

The stabilized HIFα can translocate to the nucleus where it forms a heterodimer with HIFβ, also referred to as aryl hydrocarbon receptor nuclear translocator (ARNT) (8). HIF1 then binds to the promoter/enhancer regions in the DNA (9), where it drives the expression of a wide array of hypoxia-inducible genes to augment oxygen delivery or to provide alternative pathways for energy production and cell metabolism.

The functional loss of pVHL in some RCC results in an aberrant stabilization of HIFα independent of the oxygen tension. The subsequent overexpression of proteins encoded by HIFα regulated target genes contributes to the creation of a microenvironment favorable for cell proliferation (2, 10–12).

In many tumors including RCC, the hypoxia-responsive transcription factor HIFα is overexpressed (13), and patients diagnosed with such hypoxic tumors will often have a poor clinical prognosis due to the formation of metastases and the resistance to chemotherapeutics (14). This negative prognosis may occur due to low oxygen concentrations having the capacity to induce genetic instability, leading to increased rates of mutagenesis and angiogenesis, decreased rates of apoptosis and upregulation of genes involved in the metastatic cascade (15). Suppression of the DNA damage response pathways within the hypoxic tumors may also play a critical role (9).

In addition to forming a complex with HIFα, HIF1β/ARNT also dimerizes with the aryl hydrocarbon receptor (AhR) which is known to interact with environmental pollutants such as dioxins and polycyclic aromatic hydrocarbons (PAH). PAHs are widely distributed contaminants produced as byproducts of combustion processes such as in vehicle exhaust, cigarette smoking, and charcoal grilling of food. Benzo[a]pyrene (BaP) is a classic example of PAH and is readily absorbed by inhalation, ingestion, and through the skin. As BaP is lipophilic, it can easily diffuse into cells where it binds to AhR, translocates into the nucleus and subsequently heterodimerizes with HIF1β/ARNT. This complex can then bind to the xenobiotic response elements of target genes (16) where it
acts as a transcription factor for a number of genes, which encode for enzymes involved in xenobiotic detoxification, including the cytochrome P450 (CYPs) isoforms CYP1A1 and CYP1B1 (17, 18).

The detoxification process of BaP begins with an epoxidation reaction by the mono-oxygenases CYP1A1 and CYP1B1 (phase I). The resulting metabolites (e.g., BaP-7,8-epoxide and BaP-9,10-epoxide) can be converted non-enzymatically to phenols (e.g., 3-OH BaP) or enzymatically to dihydrodiols (e.g., BaP-7,8-diOH or BaP-9,10-diOH) by epoxide hydrolase. Phenols can subsequently be converted to water-soluble sulfate or glucuronic conjugates (phase II) and dihydrodiols can be further transformed by CYP1A1 or CYP1B1 to diol epoxides (e.g., BaP-7,8-diol-9,10-epoxide (BPDE)) or conjugated by uridine diphosphate glucuronosyl transferase (UGT) (18). An unfortunate consequence of the detoxification reaction is the production of the intermediate BPDE, which can covalently bind to DNA forming highly mutagenic DNA adducts (17). When unrepaired, these lesions may result in mutations (19).

Previously, we demonstrated that exposure of cells to hypoxia markedly enhances the genetic instability caused by exogenous genotoxins and that HIF activation decreases nucleotide excision repair (NER) (20). Furthermore, we demonstrated that the kinetics of BaP metabolism is altered under hypoxia resulting in a prolonged time of exposure and a higher amount of BPDE-DNA adducts being formed (Schults et al. manuscript submitted for publication). From these initial studies in which we induced HIFα expression using chemicals or hypoxia, we observed an important link between the HIF1 mediated response pathway and the cellular response pathway that counteracts chemical carcinogens.

In the present study, we hypothesize that in naturally occurring RCC cells that have a defect in HIF regulation, the observed genetic instability may be the result of a faulty response to environmental carcinogens such as BaP. In this current report, we show that loss of VHL affects BaP-mediated genotoxic responses by inducing CYP1A1 mRNA levels, which mediated a significant change in BaP metabolism is altered under hypoxia resulting in a prolonged time of exposure and a higher amount of BPDE-DNA adducts being formed (Schults et al. manuscript submitted for publication). From these initial studies in which we induced HIFα expression using chemicals or hypoxia, we observed an important link between the HIF1 mediated response pathway and the cellular response pathway that counteracts chemical carcinogens.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

RCC4 (VHL−/−) cells, a VHL-deficient cell line and RCC4-VHL (VHL+/+), RCC4 reconstituted with VHL, were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, UK) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS; Invitrogen Breda, The Netherlands) and 1% penicillin streptomycin (P/S; Gibco, Invitrogen, Paisley, UK) at 37°C in a 5% CO2 and 20% O2 atmosphere. Cells were seeded 1 day before treatment and maintained at 37°C in a 5% CO2 atmosphere. All cells were treated with 0 or 0.1 µM BaP (Sigma) dissolved in DMSO (final concentrations did not exceed 0.5%) for 18 h. After treatment medium was removed and cells were harvested using trypsin. All samples were stored at −20°C.

QUANTITATIVE REAL-TIME PCR

Cells were washed twice with PBS and lysed with Trizol (Invitrogen). Total RNA was isolated according to the manufacturer’s instructions. The quantity of each RNA sample was spectrophotometrically assessed by a Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed using the iScript cDNA Synthesis kit (Biorad, Veenendaal, The Netherlands) starting with 1 µg of RNA. cDNA was diluted 25× in RNase free water. Real-time PCR was performed using the MiqI Single Color RT-PCR detection system (Biorad) using Sensimix Sybr Green (Quantace, London, UK), 5 µl diluted cDNA and 0.3 µM (Table 1) primers in a total volume of 25 µl. Samples were amplified under the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. PCR was checked for a-specific products by performing a melting curve analysis (65–95°C). Data were analyzed using the MiqI Software system (Biorad) and were expressed as relative gene expression (fold increase) using the 2−∆∆Ct method. The stably expressed gene cyclophilin A was included as reference.

DNA ISOLATION

Cells were harvested and resuspended in 400 µl SET/SDS (100 mM NaCl, 20 mM EDTA, 50 mM Tris, 0.5% SDS) and incubated at 37°C for 2 h. About 50 µl of DNAse-free RNase-solution (RNase A (0.1 mg/ml) and RNase T1 (1000 U/ml) in SET, incubated at 80°C for 5 min) was added and samples were incubated at 37°C for 1 h followed by adding 50 µl DNAse-free proteinase K (10 mg/ml in SET-SDS, heat-inactivated at 37°C for 30 min) and samples were incubated overnight at 37°C. After addition of 500 µl phenol/chloroform/Isoamylcohol (25:24:1), samples were rotated
for 5 min and centrifuged for 5 min at 14000 rpm. To the upper-phase, 500 µl chloroform/isoamylalcohol (24:1) was added and samples were 5 min rotated, centrifuged for 5 min (14000 rpm) and 1/30 volume NaAc (3 M, pH 5.2) was added to the upperphase. Samples were mixed for a few seconds and two volumes ethanol 100% (4°C) were added, samples were mixed and incubated at −20°C for 30 min. Samples were centrifuged for 5 min and DNA pellets were washed with ethanol 70%. DNA pellets were dried and resuspended in Milli-Q H2O. The quantity and quality of DNA was measured using the Nanodrop 1000.

**RESULTS**

To examine the influence of VHL deletion on BaP-mediated genotoxic responses, we quantified the amount of BPDE-DNA adducts in VHL-deficient RCC4 cells and compared it with the genetically wild type RCC4-VHL cells and normalized it to the total amount of DNA (Figure 1). Exposure to 0.1 µM BaP resulted in

**STATISTICAL ANALYSIS**

Results are expressed as the mean ± SE of the mean. GraphPad Prism 4 was used for statistical analysis. A two-way analysis of variance (ANOVA) with Bonferroni post hoc multiple comparison correction was used to assess differences in mRNA levels. To analyze differences in, metabolite levels, adduct levels, and repair capacity a Student’s t-test was used. Differences were considered to be statistically significant when P < 0.05.
a ~1.7-fold greater formation of the pro-mutagenic BPDE-DNA adduct in RCC4 cells, compared to RCC4-VHL cells (P < 0.05).

**CELLS DEFICIENT IN VHL HAVE CHANGES GENE EXPRESSION OF METABOLIC ENZYMES**

Since metabolism may be the underlying cause of the increase in BPDE-DNA adducts in cells deficient in *VHL*, we assessed the mRNA expression of several key phase I and II metabolic enzymes. Of the phase I enzymes responsible for the activation of BaP, CYP1A1 mRNA levels significantly differ between the two cell lines (Figure 2A). The CYP1A1 mRNA levels were ~31 and ~5.8 times higher in the RCC4 cells compared to the RCC4-VHL cells (P < 0.01), for untreated and BaP treated cells, respectively. CYP1B1 gene expression was not statistically different between the two cell lines (Figure 2B). Gene expression of four phase II enzymes responsible for the conjugation of BaP metabolites was also measured. The expression of the glutathione S-transferases GSTP1 was ~24% lower in RCC4 cells compared to RCC4-VHL cells (P < 0.05, Figure 3A). Epoxide hydrolase 1 (Figure 3B) and the UDP glucuronosyltransferases UGT1A6 (Figure 3C) showed no difference between the two cell lines. Conversely, UGT2B7 expression was ~1.5 and 1.6 times higher (DMSO and BaP treatment respectively) in RCC4 cells compared to the RCC4-VHL cells (Figure 3D).

**ABSENCE OF VHL DIRECTS BaP METABOLISM TOWARD UNFAVORABLE ACTIVATION**

To determine whether the observed differences in BaP metabolic enzymes between the two cell lines resulted in the expected detrimental changes in BaP metabolism, we assessed BaP and its metabolites BaP-9,10-diOH, BaP-7,8-diOH and 3-OH BaP by HPLC analysis with fluorescence detection. RCC4 and RCC4-VHL were exposed for 18 h to 0.1 μM BaP. DNA was isolated after 18 h and BPDE-DNA adduct levels were measured by 32P-postlabeling. Data (n = 4) are presented as mean adduct level per 10^7 nucleotides ± SE (* P < 0.05, Student’s t-test).

2.7-fold induction of BaP-9,10-diOH and 3-OH BaP, respectively, was observed in RCC4 cells compared to RCC4-VHL cells (Figures 4B,C). Furthermore, compared to RCC4-VHL cells, BaP-7,8-diOH levels, the BPDE pre-cursor, were ~2.9 times higher in RCC4 cells (Figure 4D).

**NUCLEOTIDE EXCISION REPAIR CAPACITY IS REDUCED IN VHL-DEFICIENT CELLS**

As we previously reported a downregulation of NER capacity in HIFα stabilized cells, we sought to determine whether diminished DNA repair may also play a role in the observed differences in BPDE-DNA adduct levels induction between the RCC4 and RCC4-VHL cells. Firstly, we determined the influence of DNA repair gene expression in the matched cells. The mRNA levels of the critical NER genes *XPA*, *XPC*, *ERCC1*, *ERCC5*, and *ERCC4* were not altered in the cell lines (Table 2). Secondly, as DNA repair is often not regulated at the transcription level, we used a validated modified comet assay to determine the functional NER capacity. A markedly reduced repair capacity was observed in the *VHL*-deficient cells compared to the reconstituted RCC4-VHL cells (Figure 5).

**DISCUSSION**

Previously, we demonstrated that the stabilization of HIFα by CoCl2 enhanced the carcinogenic effect of BaP in lung cancer cells and reduced repair (20). Furthermore, we demonstrated that the kinetics of carcinogen metabolism altered under hypoxic conditions. Since metabolism may be the underlying cause of the increase in BPDE-DNA adducts in cells deficient in *VHL*, we assessed the mRNA expression of several key phase I and II metabolic enzymes.
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FIGURE 3 | Loss of VHL increases UGT2B7, but decreases GSTP1 gene expression. RCC4-VHL (□) and RCC4 cells (■) were incubated with DMSO or 0.1 µM BaP for 18 h. RNA was isolated and mRNA levels were measured of (A) GSTP1, (B) EPHX1, (C) UGT1A6, and (D) UGT2B7. Data (n = 4) are presented as mean fold change ± SE (**P < 0.01, ***P < 0.001, two-way ANOVA with Bonferroni post hoc multiple comparison correction).

FIGURE 4 | Absence of VHL decreased BaP metabolism, but increased BaP metabolites. RCC4-VHL (□) and RCC4 cells (■) were incubated with 0.1 µM BaP for 18 h and extracellular unmetabolized BaP (A), BaP-9,10-diol (B), BaP-7,8-diol (C), and 3-OH BaP (D) metabolites were measured. Data (n = 5) are presented as mean area under the curve ± SE (*P < 0.05, **P < 0.01; Student’s t-test).

conditions, resulting in more BPDE-DNA adducts being formed (Schults et al. manuscript submitted for publication). The aim of the current study was to determine whether similar genetic instability mechanisms hold true in the naturally occurring VHL-deficient RCC cells. In this report, we demonstrate that the loss of VHL and via presumably the stabilization of HIFα, affects both genetic stability related processes of BaP-mediated and DNA repair capacity in RCC cells.
Deficiency resulted in increased BPDE-DNA adduct levels

Table 2 | Relative NER gene expression

| BaP | XPA | XPC | ERCC4 | ERCC5 | ERCC1 |
|-----|-----|-----|-------|-------|-------|
| 0 µM | RCC4-VHL | 1.00 ± 0.02 | 1.00 ± 0.14 | 1.00 ± 0.08 | 1.00 ± 0.10 | 1.00 ± 0.09 |
|     | RCC4   | 0.94 ± 0.05 | 0.98 ± 0.12 | 0.98 ± 0.03 | 1.16 ± 0.11 | 1.09 ± 0.06 |
| 0.1 µM | RCC4-VHL | 1.02 ± 0.04 | 1.04 ± 0.16 | 0.95 ± 0.08 | 0.98 ± 0.08 | 1.01 ± 0.02 |
|     | RCC4   | 1.15 ± 0.05 | 1.17 ± 0.19 | 0.89 ± 0.02 | 1.21 ± 0.12 | 1.17 ± 0.09 |

Statistical method used: two-way ANOVA with Bonferroni post hoc multiple comparison correction.

FIGURE 5 | NER capacity is decreased in RCC4 cells. The ability to repair BPDE-DNA adducts in RCC4-VHL and RCC4 cells were measured using the modified comet assay (23). Data (n = 4) are presented as mean fold change ± SE (*P < 0.05, Student’s t-test).

Cytochrome P450 enzymatically converts BaP into BPDE. This active metabolite subsequently binds DNA covalently forming highly mutagenic DNA adducts (17). To investigate the effect of metabolically activated BaP on RCC4 cells, the formation of BPDE-DNA adducts was determined. Our data demonstrated that the induction of the damage and its repair. Therefore, in addition to the formation of adducts, we further analyzed whether loss of VHL influences NER since the bulk of adducts are removed by this DNA repair pathway (29). Our data indicate that the loss of VHL had no effect on DNA repair gene expression. As DNA repair is often regulated in multiple levels in addition to transcriptional control, we sought to determine whether NER is functionally impaired by HIFα stabilization. To determine the functionality of NER, we used a previously validated modified comet assay, which predominantly assesses the cellular capacity in the recognition and incision phase of NER to remove bulky DNA adducts (23). A dramatically lower repair capacity was observed in the VHL-deficient RCC4 cells. Reduced NER capacity under hypoxic conditions was previously demonstrated in mouse fibroblasts (30) and in HIFα stabilized A549 cells (20). In the present study, we show that lack of VHL in cells also results in a decreased repair capacity. The reduced NER repair further explains the observed higher BPDE-DNA adduct levels in VHL-deficient RCC cells.

In this study, we provide evidence that loss of VHL presumably via the stabilization of HIF affects the BPDE-DNA adduct levels in RCC cells and is in fact a double edged sword. Firstly, the absence of VHL is associated with induced CYP1A1 mRNA levels, which mediated a significant change in BaP-7,8-diol levels among other metabolites. This could significantly induce the

in lymphocytes has been related to a high lung cancer risk (27, 28).
formation of BPDE-DNA adduct levels. Secondly, the capacity to repair DNA by NER is reduced in HIFα-stabilized cells, thereby preventing the repair of those BPDE-DNA adducts. Taken together, these data indicate that loss of VHL increases carcinogen genotoxicity in RCC in vitro and provides potential insight in the malignant progression into RCC.

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