AIP mutations impair AhR signaling in pituitary adenoma patients fibroblasts and in GH3 cells

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Abstract

Germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene predispose humans to pituitary adenomas through unknown molecular mechanisms. The best-known interacting partner of *AIP* is the aryl hydrocarbon receptor (AhR), a transcription factor that mediates the effects of xenobiotics implicated in carcinogenesis. As 75% of *AIP* mutations disrupt the physical and/or functional interaction with AhR, we postulated that the tumorigenic potential of *AIP* mutations might result from altered AhR signaling. We evaluated the impact of *AIP* mutations on the AhR signaling pathway, first in fibroblasts from *AIP*-mutated patients with pituitary adenomas, by comparison with fibroblasts from healthy subjects, then in transfected pituitary GH3 cells. The AIP protein level in mutated fibroblasts was about half of that in cells from healthy subjects, but AhR expression was unaffected. Gene expression analyses showed significant modifications in the expression of the AhR target genes *CYP1B1* and *AHRR* in *AIP*-mutated fibroblasts, both before and after stimulation with the endogenous AhR ligand kynurenine. Kynurenine increased *Cyp1b1* expression to a greater extent in GH3 cells overexpressing wild type compared with cells expressing mutant *AIP*. Knockdown of endogenous *Aip* in these cells attenuated *Cyp1b1* induction by the AhR ligand. Both mutant *AIP* expression and knockdown of endogenous *Aip* affected the kynurenine-dependent GH secretion of GH3 cells. This study of human fibroblasts bearing endogenous heterozygous *AIP* mutations and transfected pituitary GH3 cells shows that *AIP* mutations affect the AIP protein level and alter AhR transcriptional activity in a gene- and tissue-dependent manner.

Key Words

- ▶ *AIP*
- ▶ pituitary adenomas
- ▶ AhR

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Introduction

Heterozygous germline mutations of the *AIP* gene predispose humans to pituitary adenomas (Vierimaa et al. 2006). The prevalence of *AIP* mutations is about

20% in familial isolated pituitary adenomas (Beckers et al. 2013) and 3–4% in sporadic adenomas (Lecoq et al. 2015). AIP is a chaperone protein that interacts with

many molecular partners (Trivellin *et al.* 2011). One partner, the xenobiotic receptor AhR (aryl hydrocarbon receptor), is a ligand-activated transcription factor that mediates adaptive and toxic responses to environmental contaminants such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) (Schmidt *et al.* 1996). AhR also has physiological functions (Barouki *et al.* 2007), especially in cell-cycle regulation (Puga *et al.* 2009), and can play a role in tumorigenesis (Murray *et al.* 2014). Several endogenous AhR ligands have been identified (Nguyen *et al.* 2008), including tryptophan metabolites generated by the enzymes IDO (indoleamine 2,3-dioxygenase) and TDO (tryptophan dioxygenase). One such ligand, kynurenine, can promote brain tumor progression through local AhR activation (Opitz *et al.* 2011). The molecular mechanisms of AIP-dependent pituitary tumorigenesis are elusive. Recent *in vitro* studies implicate the cAMP pathway, which seems to be activated by AIP deficiency (Formosa *et al.* 2013, Tuominen *et al.* 2014), but several lines of evidence also suggest the involvement of AhR. Most pathogenic AIP mutations alter the C-terminal region of the protein, particularly the last few amino acids required for AhR interaction (Bell *et al.* 2000), resulting in loss of protein–protein interactions (Leontiou *et al.* 2008, Morgan *et al.* 2012). AhR is expressed in pituitary cells, and immunohistochemical studies have shown decreased expression of AhR or of ARNT (AhR nuclear translocator), its heterodimerization partner, in human and murine AIP-mutated pituitary adenomas (Heliövaara *et al.* 2009, Jaffrain-Rea *et al.* 2009, Raitila *et al.* 2010). Although only a slight increase in the incidence of pituitary adenomas has been noted since the Seveso accident, which released large amounts of TCDD (Pesatori *et al.* 2008), an increased prevalence of acromegaly has been observed in other highly polluted industrial areas (Cannavò *et al.* 2010), suggesting that environmental pollutants can participate in pituitary tumorigenesis, potentially through the AhR pathway.

The impact of AIP mutations on AhR signaling in patients with pituitary adenomas has not yet been investigated. Here, we explored the AhR transcriptional response to its endogenous ligand kynurenine in fibroblasts from patients with pituitary adenomas and heterozygous AIP mutations, and also in the well-characterized somatotroph GH3 cell line. We found that AIP mutations resulted in decreased AIP expression and also altered the AhR transcriptional response in both cell models, in a cell- and gene-specific manner. We have further shown that AIP mutants were able to affect kynurenine-dependent GH secretion in GH3 cells.

Materials and methods

Cell lines and treatments

Fibroblasts from four probands (patients P1 to P4) and from four sex- and age-matched controls were obtained by forearm skin biopsy. All the patients and controls gave their written informed consent for cell culture and genetic analysis, and the study was approved by Ethics Committee of Bicêtre Hospital, France. Fibroblasts were cultured in DMEM high-glucose (4.5 g/L) medium (PAA, Velizy-Villacoublay, France) supplemented with 20 mM HEPES (Life technologies, Saint Aubin, France), antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL), 2 mM glutamine (all from PAA), and 10% fetal bovine serum (FBS, Biowest, Nuaillé, France).

GH3 cells (ATCC CCL-82.1, lot 3741980) generously provided by Dr Marie Legendre (U.F. de Génétique Moléculaire, Service de Génétique et d'Embryologie Médicales, Hôpital Trousseau, Paris) were cultured in DMEM/HAM'S F-12 medium (PAA) supplemented with 20 mM HEPES, antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL), 2 mM glutamine, 2.5% FBS, and 15% donor horse serum (PAA).

All cells were cultured at 37°C in a humidified incubator with 5% CO₂. For all the experiments, the cell passage number was between 2 and 20.

Fibroblasts were treated for 8 h with 60 µM L-kynurenine (Sigma-Aldrich), with or without 10 µM 3',4'-dimethoxyflavone (Sigma-Aldrich) or 0.4 µM actinomycin D (Sigma-Aldrich), after 24 h of tryptophan deprivation in custom-made DMEM/HAM'S F-12 medium without tryptophan (Life Technologies).

Plasmid constructs

Human AIP cDNA was amplified by PCR from control (wild-type AIP-encoding vector) and proband mRNA (P1, P3, and P4 mutant-encoding vectors) using specific primers (Supplementary Table S1, see section on supplementary data given at the end of this article) in which *HindIII* and *NotI* restriction sites were introduced to facilitate cloning into the pcDNATM3.1 plasmid (Life Technologies). All plasmids were sequenced to verify nucleotide identity.

Transfection assay

GH3 cells were transfected with either the empty pcDNATM3.1 vector or with AIP-encoding vectors, using the Lipofectamine 2000 (Life Technologies) reverse-transfection protocol. After 1 day, GH3 cells were deprived

of tryptophan for 24 h and then exposed to 60 μ M L-kynurenine for 3 h. RNA and protein were extracted 48 h post-transfection as described below.

RNA interference

GH3 cells were transfected twice with 100 nM rat SMARTpool Aip siRNA or nontargeting siRNA (Dharmacon, GE Healthcare), using Lipofectamine RNAimax (Life Technologies). After 24 h of tryptophan deprivation, cells were exposed to L-kynurenine for 3 h. RNA and protein were extracted 48 h post-transfection as described below.

Reverse transcriptase-PCR (RT-PCR) and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from cells with the TRI Reagent (Molecular Research Center, Euromedex, Souffelweyersheim, France) according to the manufacturer's recommendations. After DNase treatment (Biolabs, Evry, France), RNA was reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Life Technologies). Quantitative real-time PCR (RT-qPCR) was performed as described previously (Le Billan et al. 2015). The primer sequences for the genes analyzed by RT-qPCR are shown in [Supplementary Table S2](#).

Western blot (WB)

Total protein extracts were prepared from cells lysed in lysis buffer, 1X Triton X100 and 1X protease inhibitor (Sigma-Aldrich). Immunoblots were incubated for 1 h at room temperature (RT) in 5% milk-Tris buffer saline/0.1% Tween 20 before incubation overnight with anti-AIP, anti- β -actin, anti-AhR, anti- α -tubulin, anti-CYP1B1, or anti-GAPDH antibodies (antibody sources and dilutions are shown in the [Supplementary Table S3](#)). Blots were then incubated with an IRDye 800-conjugated affinity-purified anti-mouse IgG second antibody (1:15,000 dilution, Perbio Science, Brebières, France) and an IRDye 680-conjugated affinity-purified anti-rabbit IgG second antibody (1:15,000 dilution, Perbio Science) for 1 h at RT. Proteins were visualized with an Odyssey-Fc apparatus (LI-COR).

Cell proliferation analysis

Cell proliferation tests were performed using the WST1 assay (Roche) according to the manufacturer's recommendations. GH3 cells were cultured in 96-well

plates, transfected with either the empty pcDNATM3.1 vector or wild-type AIP-encoding vectors using the Lipofectamine 2000 (Life Technologies) reverse-transfection protocol. Cells were deprived of tryptophan and exposed to 60 μ M L-kynurenine for 48 h. Optical densities were measured 4 h after the addition of WST1 solution (10 μ L per well) by spectrophotometry (Viktor, PerkinElmer).

Hormone assays

GH3 cells were cultured in 24-well plates, transfected with either the empty pcDNATM3.1 vector or AIP-encoding vectors using the Lipofectamine 2000 (Life Technologies) reverse-transfection protocol or transfected twice with 100 nM rat SMARTpool Aip siRNA or nontargeting siRNA (Dharmacon, GE Healthcare), using Lipofectamine RNAimax (Life Technologies). Cells were deprived of tryptophan and exposed to 60 μ M L-kynurenine for 48 h. After 48 h, GH secreted in the medium was determined by EIA as described previously (Steyn et al. 2011), using antibodies listed in the [Supplementary Table S3](#). Values are reported in terms of rGH-RP2. The detection limit was 0.038 ng/mL and the intra- and interassay coefficients of variation were 3.2% and below 8.75%, respectively.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Differences between groups were analyzed by two-way ANOVA followed by a multiple comparisons post-test, or with the nonparametric Mann-Whitney test (Prism software, GraphPad, CA, USA). *P* values <0.05 were considered statistically significant.

Results

Clinical and genetic characterization of AIP-mutated patients with pituitary adenomas

[Table 1](#) shows the clinical features of the four patients studied here (P1 to P4). As expected in the case of AIP mutation, the patients were young at diagnosis and had macro- or giant, GH-secreting or GH and PRL-cosecreting adenomas. Two of the three patients with somatotropinomas were giants. The AIP mutations were located throughout the coding sequence ([Fig. 1A](#)). Patient 1 (M1) had a missense mutation in the start codon that abrogated protein expression. The frameshift mutation (M2) in patient 2 and the nonsense mutation (M4) in patient 4 theoretically resulted in the expression

Table 1 Clinical characteristics of the four *AIP* mutation positive patients with pituitary adenomas.

Patient	<i>AIP</i> mutation	Sex	Age at diagnosis (years)	Clinical presentation	Adenoma type	IGF-I and/or PRL at diagnosis (ng/mL)	Adenoma size
1	c.2T>C	F	42	Galactorrhea, sweating	GH + PRL	124/40	MA
2	p.? c.350delG p.(Gly117Alafs*39)	F	30	Primary amenorrhea	PRL	–/7460	GA
3	c.805–825dup	M	14	Gigantism and hypopituitarism	GH	766/–	MA
4	p.(Phe269–His275dup) [†] c.910C>T	M	20	Gigantism and hypopituitarism	GH	617/–	GA
	p.(Arg304*) [†]						

The accession number used for the *AIP* transcript is NM_003977.2.

fs, frameshift; F, female; M, male; GH, growth hormone; PRL, prolactin; IGF-I, insulin-like growth factor I; MA, macro-adenoma (10 mm < diameter < 40 mm); GA, giant adenoma (≥40 mm diameter); *AIP*mut, *AIP* mutation; N/A, not available.

[†]Mutations encountered in FIPA patients.

Figure 1

AIP expression in fibroblasts and pituitary adenoma of *AIP*-mutated patients. (A) The four *AIP* mutations studied here were located throughout the coding sequence and had different predicted effects on the AIP protein. (B) AIP mRNA levels measured by RT-qPCR were lower in the fibroblasts of patients 1 and 2 (M1 and M2) than in controls. AIP transcript levels in M3 and M4 fibroblasts were similar to control values. Results of RT-qPCR are expressed as the mean percentage of control $\pm$ S.E.M. of at least six independent determinations performed in duplicate. (C) WB analysis showed lower AIP protein expression in all the patients' fibroblasts than in controls. (D) Quantitative analysis of six different WB experiments confirmed the decrease in AIP protein expression in mutated fibroblasts. Specific signals for AIP were normalized by the infrared fluorescence of β -actin. Results are shown as the AIP/ β -actin ratio and as percentage of control $\pm$ S.E.M. (E) WB analysis revealed the absence of AIP protein in the pituitary adenoma of patient 1. For all experiments, values obtained with fibroblasts from the four controls were pooled and arbitrarily set at 100%. * P < 0.05, *** P < 0.001. M, mutation; bp, base pair; FKBP-PPI, FK506 binding protein-peptidyl-prolyl-isomerase; TPR, tetratricopeptide repeats; AA, amino acid; kDa, kiloDalton.

of a protein truncated of the entire C-terminal domain or only the last few amino acids, respectively. The duplication (M3) in patient 3 resulted in a protein with a longer tetratricopeptide repeats (TPR) domain. As AIP is ubiquitously expressed, we used fibroblasts obtained by skin biopsy to study the expression and molecular consequences of the AIP mutants.

AIP and AhR expression in the patients' fibroblasts

As shown in Fig. 1B, AIP mRNA expression was significantly lower (~50%) in M1 and M2 fibroblasts than in controls. AIP transcript levels in M3 and M4 fibroblasts were similar to control values. We examined whether nonsense-mediated mRNA decay (NMD) could explain this low mRNA level in M2 fibroblasts. NMD is a translation-coupled mechanism that eliminates mutated mRNAs that contain premature translation-termination codons located before the last exon. Indeed, as M2 resulted in a premature termination codon located in the third exon, NMD may account for the M2 AIP mRNA degradation and thus for the lack of truncated M2 protein (Silva et al. 2009). The presence of heterozygous AIP mutation in genomic DNA from M2 fibroblasts was confirmed, but direct cDNA sequencing failed to detect mutant transcripts (data not shown). Treatment of M2 fibroblasts with emetine, a potent NMD inhibitor, stabilized the mutant mRNA expressed from the defective allele and significantly increased the total amount of AIP mRNA, as measured by RT-qPCR (Supplementary Fig. S1).

AIP protein expression was lower (~50%) in all four patients' fibroblasts than in controls (Fig. 1C and D). No truncated or aberrant proteins were detected by WB with an antibody recognizing the N-terminal part of AIP. Interestingly, AIP protein expression was completely abolished in the pituitary adenoma of patient 1 (Fig. 1E). The sub-normal AIP protein expression in patients' fibroblasts, together with the lack of truncated forms, prompted us to examine the expression of AIP mutants in a translation-coupled-to-transcription assay. As expected, the M1 mutant could not be translated. We confirmed that the M3 and M4 mutants could be translated *in vitro*, into a longer and a shorter protein, respectively (Supplementary Fig. S2).

Analysis of AhR mRNA (Fig. 2A) and protein (Fig. 2B and C) showed that the expression levels in the patients' fibroblasts were similar to control values, with variable protein expression levels in multiple WB experiments (Fig. 2C). ARNT transcript levels were also similar in AIP-mutated and control fibroblasts (data not shown).

Figure 2 AhR expression in the fibroblasts of AIP-mutated patients and controls. (A) AhR transcript levels were similar in mutated and control fibroblasts, as determined by RT-qPCR. Results are expressed as the mean percentage of control $\pm$ S.E.M. of at least six independent determinations performed in duplicate. Values obtained with fibroblasts from the four controls were pooled and arbitrarily set at 100%. (B) AhR protein expression in WB experiments did not differ between AIP-mutated fibroblasts and controls. The positive control on this representative WB corresponds to protein extracts of HEK293T cells transfected or not transfected with a human AhR expression vector. (C) Quantitative analysis of six different WB experiments confirmed this result. Specific signals for AhR were normalized by the infrared fluorescence of α -tubulin. Data are presented as the AhR/ α -tubulin ratio and as the percentage of control $\pm$ S.E.M. Values obtained with fibroblasts from the four controls were pooled and arbitrarily set at 100%.

Thus, heterozygous AIP mutations do not alter intracellular AhR or ARNT levels in this cell model.

Expression of AhR target genes in AIP-mutated fibroblasts

To explore the impact of AIP mutations on AhR signaling, we studied the expression of CYP1B1, a prototypical

Figure 3

CYP1B1 expression in fibroblasts from *AIP*-mutated patients and controls. (A) Fibroblast *CYP1B1* expression was induced by kynurenine, as shown by RT-qPCR. 3',4'-dimethoxyflavone, an AhR antagonist, abolished this effect of kynurenine. Cells were treated with 60 μM kynurenine, with or without 10 μM 3',4'-dimethoxyflavone, for 8 h. (B) Actinomycin D also reversed the effect of kynurenine on fibroblast *CYP1B1* mRNA expression, as shown by RT-qPCR. Cells were treated with 60 μM kynurenine, with or without 0.4 μM actinomycin D, for 8 h. (C) Kynurenine induced *CYP1B1* expression in a time-dependent manner, peaking after 8 h of treatment. Cells were incubated for various times in the presence or absence of 60 μM kynurenine. (D) Kynurenine induced *CYP1B1* expression in a concentration-dependent manner, peaking at 25 μM. Cells were incubated with various kynurenine concentrations for 8 h. (E) *CYP1B1* transcript levels measured by RT-qPCR were lower in the fibroblasts of patients 2 and 4 than in controls, the difference reaching statistical significance only after exposure to 60 μM kynurenine for 8 h. (F) RT-qPCR results were confirmed by WB. Results of RT-qPCR are expressed as the mean percentage of control ± s.e.m. of at least four (A–D) or six (E) independent determinations performed in duplicate; values of vehicle-treated cells being arbitrarily set at 100%. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Kynu, kynurenine; 3,4-DMF, 3',4'-dimethoxyflavone; ActD, actinomycin D.

AhR target gene that encodes a xenobiotic-metabolizing enzyme. As shown in Fig. 3A, B, C and D, fibroblasts exposure to the AhR ligand kynurenine resulted in a three- to four-fold increase in *CYP1B1* transcript levels, as measured by RT-qPCR. Both 3',4'-dimethoxyflavone (3,4-DMF), an AhR antagonist (Fig. 3A), and actinomycin D (ActD) (Fig. 3B) totally abolished the kynurenine-dependent increase in *CYP1B1* transcript levels, indicating direct transcriptional control of *CYP1B1* expression by AhR. This transcriptional response was time- and concentration-dependent (Fig. 3C and D), with maximal induction after 3–8 h and at a kynurenine concentration of 25 μM. Basal *CYP1B1* transcript levels tended to be lower than control values in *AIP*-mutated fibroblasts, especially those from patients 2 and 4 (Fig. 3E). After kynurenine exposure, *CYP1B1* transcript and protein levels were significantly lower in M2 and M4 fibroblasts than in controls, while both transcript and protein levels in M1 and M3 cells were similar to control values (Fig. 3E and F). Interestingly, the *AIP* mutations had different impacts on the expression of *AhRR* (aryl hydrocarbon receptor repressor), another AhR-regulated gene.

Basal and kynurenine-induced *AhRR* mRNA levels were both higher in mutated fibroblasts than in controls (Fig. 4). Together, these results suggest that basal and ligand-induced transcriptional activation of AhR is modified in *AIP*-mutated fibroblasts, in a gene-specific manner.

Effect of *AIP* overexpression on *Cyp1b1* mRNA expression in GH3 cells

As *AIP* mutations predispose humans to pituitary adenomas, we examined whether these mutations altered AhR signaling in the somatotroph GH3 cell line. As M2 *AIP* mRNA is degraded through an NMD mechanism, only the M1, M3, and M4 mutants were transfected into GH3 cells in order to examine AhR target gene expression. WB analysis confirmed that GH3 cells were successfully transfected (Fig. 5A). First, we used RT-qPCR to measure *Cyp1b1* transcript levels after transfection of human wild-type (WT) and mutant *AIP*. Overexpression of neither WT nor mutant *AIP* significantly altered basal expression of the *Cyp1b1* gene (Fig. 5B). Exposure to kynurenine induced a three-fold increase in *Cyp1b1* mRNA

Figure 4

AhRR expression in fibroblasts from *AIP*-mutated patients and controls. Basal and kynurenine-induced *AhRR* mRNA expression was significantly higher in *AIP*-mutated fibroblasts than in controls, as measured by RT-qPCR. Results are expressed as the mean percentage of control $\pm$ S.E.M. of at least six independent determinations performed in duplicate. Values obtained with fibroblasts from the four controls were pooled, and values for untreated cells were arbitrarily set at 100%. **** $P < 0.0001$.

expression. Cells transfected with WT AIP showed a larger increase in *Cyp1b1* mRNA on kynurenine exposure than those transfected with either the empty vector (EV) or the mutant AIP vectors (Fig. 5B). Thus, overexpression of WT AIP, but not the AIP mutants, increased kynurenine-induced AhR target gene expression.

Effect of *Aip* knockdown on *Cyp1b1* mRNA expression in GH3 cells

To further investigate the influence of the AIP expression level on AhR signaling, we measured *Cyp1b1* transcript levels after inhibiting endogenous *Aip* gene expression with small interfering RNA (siRNA). As shown in Fig. 5A and C, a 70% decrease in endogenous AIP mRNA and a 60% decrease in endogenous AIP protein were observed, in both the absence and presence of kynurenine. Even partial silencing of *Aip* significantly reduced the increase in *Cyp1b1* transcript levels after kynurenine exposure (Fig. 5D). Basal *Cyp1b1* transcript levels were reduced by *Aip* knockdown but the difference did not reach statistical significance ($P = 0.05$). Together, these results show that a reduction in AIP expression impairs AhR transcriptional activity in GH3 cells.

Impact of AIP on GH3 cells proliferation and GH secretion

We observed that in GH3 cells transfected with the empty vector, expressing only endogenous AIP protein, kynurenine

treatment decreased cell proliferation (Supplementary Fig. S3). In untreated cells, transient transfection of WT AIP reduced cell proliferation, compared with the empty vector control. Kynurenine treatment reinforced this effect of WT AIP overexpression (Supplementary Fig. S3).

We further performed GH secretion assays in transfected GH3 cells after kynurenine stimulation. We observed a decrease in GH secretion in cells transfected with AIP mutants compared with cells transfected with either empty vector or WT AIP (Fig. 6A). A similar decrease in GH secretion was observed after *Aip* knockdown with siRNA in kynurenine-treated GH3 cells (Fig. 6B).

Discussion

We describe for the first time the functional impact of human AIP mutations on AhR signaling, using fibroblasts from patients with heterozygous AIP mutations. Previously, the role of AIP in AhR signaling had only been analyzed in murine cell lines overexpressing exogenous AIP, and none of the AIP mutations previously identified in patients with pituitary adenomas had been examined for their impact on the AhR pathway. The AIP mutations studied here have already been reported (Vierimaa et al. 2006, Personnier et al. 2011, Cazabat et al. 2012) and are considered pathogenic (Beckers et al. 2013, Hernández-Ramírez et al. 2015a). Two of these mutations (M3 and M4) have been reported in patients with familial isolated pituitary adenomas. Interestingly, the four mutations studied here had different predicted impacts on AIP protein expression, two of them (M2 and M4) potentially leading to a truncated protein lacking the C-terminal domain required for AIP–AhR interaction.

We found that the heterozygous AIP mutations resulted in decreased AIP protein expression, by comparison with AIP wild-type fibroblasts. In the case of mutations M1 and M2, this is easily explained by the loss of the translation initiation codon and by an NMD mechanism, respectively. Regarding mutations M3 and M4, the reduced AIP expression and the nonexpression of a truncated AIP protein could be linked to a shorter protein half-life due to enhanced degradation (Hernández-Ramírez LC et al. 2015b). The absence of AIP protein in the pituitary adenoma of patient 1 (M1) points to a ‘second hit’ (Knudson’s hypothesis (Knudson 1971)), which could be a loss of heterozygosity, as observed elsewhere in several AIP-mutated tumors (Lecoq et al. 2015).

Interestingly, this decrease in endogenous AIP protein expression did not affect the AhR expression level. As AIP has been shown to protect the ligand-free form

Figure 5

Cyp1b1 expression in GH3 cells after overexpression of wild-type and mutant AIP and knockdown of endogenous *Aip*. (A) WB analysis of AIP expression in GH3 cells transfected with WT AIP, mutant AIP, nontargeting siRNA (NT siRNA), or *Aip* siRNA. The WB shows overexpression of WT AIP, lack of the truncated AIP mutants, and successful knockdown of endogenous rat *Aip* by *Aip* siRNA. Protein extraction was performed 48 h post-transfection. (B) Kynurenine-induced *Cyp1b1* expression in GH3 cells, as measured by RT-qPCR. Cells overexpressing wild-type AIP exhibited a large increase in *Cyp1b1* transcript levels after kynurenine exposure than cells transfected with an empty vector ($P < 0.0001$) or mutant AIP ($P < 0.0001$ for WT AIP vs M1 and WT AIP vs M3 and $P < 0.001$ for WT AIP vs M4). GH3 cells were transfected with either the empty pcDNATM3.1 vector or AIP-encoding vectors. After 1 day, cells were deprived of tryptophan for 24 h and then exposed to 60 μ M L-kynurenine for 3 h. RNA extraction was performed 48 h post-transfection. (C) Transfection of GH3 cells with *Aip* siRNA for 48 h resulted in successful knockdown of endogenous rat *Aip*, with a 70% decrease in *Aip* transcript levels in RT-qPCR experiments. (D) Knockdown of endogenous *Aip* attenuated the increase in *Cyp1b1* transcript levels after kynurenine exposure. Cells were transiently transfected with *Aip* siRNA and nontargeting siRNA. After 48 h, cells were left either untreated or treated with 60 μ M kynurenine for 3 h before RNA extraction. Changes in *Cyp1b1* mRNA expression were detected by RT-qPCR. Results are expressed as the mean percentage of control $\pm$ S.E.M. of at least four (B) or six (C–D) independent determinations performed in duplicate, values for untreated cells transfected with an empty vector (B) or with nontargeting siRNA (C–D) being arbitrarily set at 100%. *** $P < 0.001$, **** $P < 0.0001$. EV, empty vector; WT, wild-type; NT, nontargeting.

of murine AhR against ubiquitination (Meyer et al. 1999, Kazlauskas et al. 2000, LaPres et al. 2000), a decrease in AhR protein levels might have been expected in AIP-mutated fibroblasts. This unexpected finding might be explained by different roles of AIP in human vs mouse AhR complexes (Ramadoss et al. 2004). In addition, other chaperone proteins important for AhR stabilization, such as HSP90, might compensate for the AIP deficiency (Chen et al. 1997).

To explore the impact of AIP mutations on AhR signaling, we studied the expression of *CYP1B1*, a prototypical AhR target gene, before and after kynurenine exposure. Kynurenine was chosen to stimulate AhR because this oncometabolite, being an endogenous AhR ligand, is likely to be devoid of the toxic effects of TCDD and has been shown to activate AhR in tumor cells (Opitz et al. 2011). We confirm that kynurenine also activates AhR-dependent *CYP1B1* transcription in patients' fibroblasts and in pituitary cells, at concentrations as low as 25 μ M. However, we have chosen the 60 μ M concentration which corresponds to the concentration measured in the media of human glioma cells and which was able to stimulate

the AhR pathway in these cells (Opitz et al. 2011). Interestingly, we have found that the tryptophan-metabolizing enzyme IDO2 required for kynurenine synthesis is also expressed in somatotroph adenomas (not shown). Thus, locally produced kynurenine could play a role in AIP-related pituitary tumorigenesis but its pituitary production remains to be demonstrated.

We show for the first time that heterozygous human AIP mutations modify both basal and ligand-induced AhR transcriptional activity in human fibroblasts. This effect is particularly observed after kynurenine exposure for *CYP1B1*, which is similar to what is reported in other studies assessing the *in vitro* transcriptional activity of AhR (Ma et al. 1997, Pollenz et al. 2005) and with other transcription factors such as nuclear receptors (Laenger et al. 2009). The differential impact of AIP mutations on *CYP1B1* transcription is intriguing. The exact molecular mechanisms by which these mutations alter the transcriptional regulation of *CYP1B1* by AhR is currently unknown. In the M2 and M4 cell lines, AIP mutations lead in theory to truncation of the C-terminal part of the AIP protein required for interaction with AhR.

The resulting mutant proteins could act as dominant negatives of the WT AIP protein by interfering with the AhR-dependent transcription. Nevertheless, the existence of these truncated proteins is questionable since we were unable to detect them by Western blot analysis, but this does not formally rule out their transient expression. Alternatively, the M2 and M4 mutations may simply result in the absence of mutated AIP protein and thus lead to AIP haploinsufficiency, which could be responsible for the observed impact on AhR target gene transcription. Previous studies have shown that AIP translocates to the nucleus together with AhR in human cells (Ramados *et al.* 2004), and that variations in AIP expression may enhance or repress AhR transcriptional activity (Ma *et al.* 1997, Carver *et al.* 1998, Hollingshead *et al.* 2004). Thus, AIP deficiency might modify AhR nuclear translocation and/or AhR binding to ARNT and coregulators. We found that different *AIP* mutations had different impacts on *CYP1B1* and *AhRR* transcription, thus underlining the complexity of AhR signaling; indeed, this transcription factor can bind several response elements and recruit various coactivators/repressors in the regulatory sequences of its target genes (Hankinson 2005).

Several lines of evidence suggest a functional role of the AhR signaling pathway in the somatotroph cells. First, using the well-characterized somatolactotroph GH3 cells, we found that the overexpression of WT AIP, but not the AIP mutants, enhanced AhR transcriptional activity, while knockdown of endogenous *Aip* gave the opposite results. This suggests that AIP deficiency in these pituitary cells may lead to downregulation of AhR transcriptional activity. Secondly, activation of AhR signaling by kynurenine had antiproliferative action in this cell model. Finally, unlike cAMP pathway activation by forskolin (Formosa *et al.* 2013), AhR activation by kynurenine did not modify basal GH secretion from untransfected GH3 cells expressing normal amounts of endogenous AIP (not shown). However, both AIP mutants and *Aip* knockdown decreased GH secretion from kynurenine-treated GH3 cells. A similar observation has been previously reported after *Aip* knockdown in GH3 cells exposed to forskolin and IBMX (Formosa *et al.* 2013). Thus, AIP haploinsufficiency, both after cAMP pathway and AhR pathway activation, decreases GH secretion from GH3 cells. These results seem paradoxical considering the role of *AIP* mutations in somatotroph tumorigenesis. Nevertheless, similarly to what has been recently reported for *ARMC5* mutations (Assié *et al.* 2013), we can hypothesize that the inactivation of *AIP* might be, *per se*, associated with a partial dedifferentiation of pituitary

Figure 6

Effect of AIP mutants and *Aip* knockdown on kynurenine-induced GH secretion in GH3 cells. (A) GH secretion was significantly lower in kynurenine-treated GH3 cells transfected with M1, M3, and M4 mutants. Cells were transfected with either the empty pcDNATM3.1 vector or AIP-encoding vectors, deprived of tryptophan and exposed to 60 μ M L-kynurenine for 48 h. GH concentrations were measured in the medium 48 h post-transfection. Results are expressed as the mean percentage of control $\pm$ S.E.M., values for untreated cells transfected with an empty vector being arbitrarily set at 100%. (B) *Aip* knockdown in GH3 cells induced a decrease in GH secretion after a 48 h exposure to kynurenine. Cells were transiently transfected with *Aip* siRNA and nontargeting siRNA. After 48 h, cells were left either untreated or treated with 60 μ M kynurenine for 48 h before GH secretion analysis. Results are expressed as the mean percentage of control $\pm$ S.E.M., values for cells transfected with nontargeting siRNA being arbitrarily set at 100%. * P < 0.05, ** P < 0.01, *** P < 0.001. EV, empty vector; WT, wild-type; NT, nontargeting.

somatotroph cells leading to altered capacity of mutated cells to produce GH. The increased proliferation rate of somatotroph cells bearing *AIP* mutations (Leontiou *et al.* 2008) leads to tumor formation and *in fine* to GH excess.

In conclusion, in patients' fibroblasts bearing endogenous heterozygous *AIP* mutations and in transfected pituitary GH3 cells, *AIP* mutations affect AhR transcriptional activity in a gene- and tissue-dependent manner. How altered AhR signaling contributes to pituitary tumorigenesis remains to be further investigated.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0041>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

A L L, M L, P C and P K contributed to the conception and design of the research; A L L, S V, M H and P Z performed the experiments; J B performed AIP sequencing; A B obtained fibroblasts from the patients' skin biopsies; J Y, P C and P K referred the patients; A L L, S V, M H and P K analyzed the data; A L L, S V, M L, P C and P K interpreted the results of the experiments; A L L, S V and P K prepared the figures; A L L and P K drafted the manuscript; A L L, S V, M L, P C and P K edited and revised the manuscript; A L L, S V, J B, J Y, M L, P C and P K approved the final version of the manuscript.

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