Celecoxib reduces glucocorticoids in vitro and in a mouse model with adrenocortical hyperplasia

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Abstract

Primary pigmented nodular adrenocortical disease (PPNAD), whether in the context of Carney complex (CNC) or isolated, leads to ACTH-independent Cushing’s syndrome (CS). CNC and PPNAD are caused typically by inactivating mutations of PRKAR1A, a gene coding for the type 1a regulatory subunit (R1α) of cAMP-dependent protein kinase (PKA). Mice lacking Prkar1a, specifically in the adrenal cortex (AdKO) developed CS caused by bilateral adrenal hyperplasia (BAH), which is formed from the abnormal proliferation of fetal-like adrenocortical cells. Celecoxib is a cyclooxygenase 2 (COX2) inhibitor. In bone, Prkar1a inhibition is associated with COX2 activation and prostaglandin E2 (PGE2) production that, in turn, activates proliferation of bone stromal cells. We hypothesized that COX2 inhibition may have an effect in PPNAD. In vitro treatment of human cell lines, including one from a patient with PPNAD, with celecoxib resulted in decreased cell viability. We then treated AdKO and control mice with 1500 mg/kg celecoxib or vehicle. Celecoxib treatment led to decreased PGE2 and corticosterone levels, reduced proliferation and increased apoptosis of adrenocortical cells, and decreased steroidogenic gene expression. We conclude that, in vitro and in vivo, celecoxib led to decreased steroiogenesis. In a mouse model of PPNAD, celecoxib caused histological changes that, at least in part, reversed BAH and this was associated with a reduction of corticosterone levels.

Key Words
- cAMP
- prostaglandin E2
- celecoxib
- primary pigmented nodular adrenocortical disease (PPNAD)
- adrenal tumors

Introduction

Primary pigmented nodular adrenocortical disease (PPNAD) leads to adrenocorticotropic hormone (ACTH)-independent Cushing’s syndrome (CS) (Carney et al. 1985, Stratakis 2007) and is caused typically by inactivating mutations of PRKAR1A, a gene coding for the type 1a regulatory subunit (R1α) of cAMP-dependent protein kinase (PKA) (Kirschner et al. 2000, Stratakis 2008). Prkar1a mice with the gene entirely knocked out (KO) specifically in the adrenal cortex (AdKO) developed bilateral adrenal hyperplasia (BAH) that formed from resurgence and
proliferation of fetal-like adrenocortical cells (FLACs) (Sahut-Barnola et al. 2010, de Joussineau et al. 2012). These mice developed a late-onset CS that was more prominent in female animals, as is true for humans with PPNAD (Bertherat et al. 2009). FLACs have since been found in the adrenal cortex of humans with ACTH-independent CS and various forms of BAH (Carney et al. 2010, 2011, 2012) and are currently considered responsible for PPNAD and related disorders (Almeida & Stratakis 2011).

Similarly, in bone, cells of multipotent nature were found to be responsible for yet another benign neoplasm that is occasionally associated with PPNAD in the context of Carney complex (CNC), osteochondromyxoma (Carney et al. 2001). In both the Prkar1a heterozygous mouse (Kirschner et al. 2005) and in the double heterozygous mice deficient in both Prkar1a and Prkaca (Prkar1a<sup>+/−</sup> Prkaca<sup>+/−</sup>) (Tsang et al. 2010), the main catalytic subunit of PKA, abnormal proliferation of adult bone stromal cells (aBSCs) led to multiple tumors and fibrous dysplasia-like lesions. In both these animal models, aBSCs led to multiple tumors and fibrous dysplasia-like lesions. In both these animal models, aBSCs led to multiple tumors and fibrous dysplasia-like lesions.

Celecoxib, an FDA-approved drug for use in humans and effective inhibitor of the prostaglandin-endoperoxide synthase 2 (PTGS2) enzyme (also known as cyclooxygenase 2 (COX2)) has been used with the intent to decrease neoplastic growth and/or proliferation in a variety of tumors (Bertagnolli 2006, Daikoku et al. 2014, Yokouchi et al. 2014, Kurtova et al. 2015). We hypothesized that celecoxib might also be effective in decreasing the proliferation of adrenocortical cells in adrenal lesions derived from FLACs, and if true, possibly also decrease glucocorticoid production from affected glands. Our data support this hypothesis, as both in vitro and in vivo celecoxib decreased steroidogenesis and reversed, at least in part, BAH in the treated animals.

**Materials and methods**

**Animal protocol**

AdKO mice have been described previously (Sahut-Barnola et al. 2010). AdKO mice were maintained on a mixed genetic background (C57BL/6/129Sv). There were two groups used in the 6-month-long experiment: celecoxib-treated and vehicle. Celecoxib-treated mice (all female, n = 5) were treated with celecoxib 1500 p.p.m. mixed in NIH-31 diet; the vehicle (control) group of AdKO mice (all female, n = 4) were on the same NIH-31 diet. Treatment started at the age of 3 months and ended at 9 months of age, because this is the time that these animals develop clinically evident BAH (Sahut-Barnola et al. 2010, de Joussineau et al. 2012). We also used only female mice because the BAH phenotype is mostly apparent in them, whereas it is not as penetrant in male mice. Following treatment, mice were euthanized and adrenals harvested for histopathologic and molecular studies. All analyses were done on age-matched mice; animals were from the same litters to avoid strain effects.

All animal studies were performed under protocol ASP12-033 and were approved by and conducted in accordance with the Eunice Kennedy Shriver National Institute for Child Health and Human Development (NICHD) Institutional Animal Care and Use Committee.

**Genotyping**

PCR was used for genotyping. Three primers (5'-AGC-TAGCTTGCTGAGCTA-3', 5'-AAGCAGGAGCTAT-TAGTTTAT-3', and 5'-CATCCCATCTCCTATCCCCCTTT-3') were used for Prkar1a genotyping; the WT allele generates a 250 bp fragment while the Prkar1a<sup>loxP</sup> allele generates a 320 bp product.

**Cell cultures and cell counting**

Human adrenal cell lines H295R and CAR47 (Nesterova et al. 2008), and the embryonic kidney cell line HEK293 were cultured as monolayers on pretreated T75 flasks. CAR47 was cultured, as previously described (Nesterova et al. 2008) in DMEM (Life Technologies) with 20% fetal bovine serum (FBS) (Gemini Bio-products, West Sacramento, CA, USA) and 1% anti-anti (Life Technologies). Cell numbers were counted using the Luna Automated Cell Counter (Logo Biosystems, Annandale, VA, USA) following Trypan blue staining.

**MTT cell viability assay**

The Vybrant MTT Cell Proliferation Assay Kit (Life Technologies) was used to examine the effect of celecoxib on cell growth rates in each of the three cell lines: CAR47,
H295R, and HEK293. Cells were seeded at 5000 cells/well in 96-well plate and cultured for 48 h. Cells were treated with different concentrations of celecoxib: 1–10 μM diluted in DMSO for 24 h. Cells without treatment were used as control whereas cells treated with DMSO were used as vehicle control. Cell numbers after celecoxib treatment were determined by MTT assay as a representative of cell growth rates. For each well, 10 μl MTT stock solution was added to 100 μl fresh medium and incubated at 37 °C for 4 h. Then 100 μl of SDS–HCl solution was added to each well and incubated at 37 °C for 16 h in a humidified chamber. Absorbance was read at 570 nm.

Cortisol measurements

DetectX Cortisol Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA) was used to exam cortisol concentrations in H295R cell culture medium with and without celecoxib treatment. In each well of a 96-well plate, 5000 cells were seeded. Cells were treated with different concentrations of celecoxib: 1–10 μM diluted in DMSO for 24 h. Cells without treatment were used as control and cells treated with DMSO were used as vehicles. After 24 h, cell culture medium was collected and used to measure cortisol concentrations. Briefly, and following manufacturer’s instructions, 50 μl of samples or standards were pipetted into wells in the plate, followed by 25 μl of DetectX cortisol conjugate and 25 μl DetectX cortisol antibody. The plate was incubated at room temperature for 1 h, then washed four times with a wash buffer. After 100 μl of TMB substrate was added to each well and incubated at room temperature for 30 min. Next, 50 μl of the stop solution was added to each well. Optical density generated from each well was read in a plate reader at 1:400, Cell Signaling Technology, Danvers, MA, USA) using the Rabbit ImmPRESS Kit (Vector Laboratories, Burlingame, CA, USA), following manufacturers protocol. Staining was visualized using ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories). Coverslips were mounted onto the stained sections using VectaMount AQ Aqueous Mounting Media (Vector Laboratories). Slides were examined using the Leica DMRX microscope. Images were acquired with the DP72 camera (Olympus) using the CellSans Software (Olympus).

Corticosterone measurements

A mouse corticosterone ELISA (ALPCO Diagnostics, Salem, NH, USA) was used to measure serum corticosterone levels in age-matched 9-month-old celecoxib-treated and control AdKO mice, as per the manufacturer’s instructions.

Studies of the glucocorticoid receptor in mouse adrenal glands

RNA studies Frozen adrenals were disrupted in lysis buffer and total mRNAs were extracted using an RNA extraction kit (Macherey–Nagel, Bethlehem, PA, USA) according to manufacturer’s instructions. Total RNAs (1 μg) were reverse-transcribed by Moloney murine leukaemia virus reverse transcriptase (Promega Corp.) according to the manufacturer’s instructions. Semi quantitative PCR was stopped at the exponential phase. Primers that were used in this study: glucocorticoid receptor (GR), 5′-CCCTTCCCTTCCAGATTAG-3′ and 5′-CAGCTCTTTT- CAGGACCTTCG-3′, 599 bp; β-actin, 5′-CGTGGCGCCCGC- CTAGGCCCA-3′ and 5′-TTGCGCTTAGGTTCAGG GGG-3′, 242 bp.

Protein studies: western blot and immunostaining Protein was extracted by disruption of frozen adrenals in RIPA buffer in the presence of protease inhibitors (complete mix, Roche). Proteins (30 μg) were loaded on a 8% SDS–PAGE gel, transferred onto nitrocellulose and detected with antibody to GR (1:200, GR(M-20) SC-1004, Santa Cruz Biotechnology). Expression of these proteins was normalized to expression of β-actin and signals were analyzed with Quantity One (Bio-Rad).

Paraffin-embedded AdKO adrenal sections underwent deparaffinization by Histoclear and a graded series of ethanol washes. Followed by immunodetection of GR (1:2000, GR(M-20) SC-1004, Santa Cruz Biotechnology) was carried out on an Intavis InSitu Pro VSi automated processor (Intavis AG, Cologne, Germany). Epitopes were

Haematoxylin and eosin and other immunohistochemistry

Adrenal glands that were dissected from mice were fixed in 10% neutral buffered formalin, embedded into paraffin blocks and sectioned (5 μm) and onto glass slides, following standard procedures by Histoserv, Inc. (Germantown, MD, USA). Histological staining using haematoxylin and eosin staining was also performed (Histoserv, Inc.). Immunohistochemistry (IHC) was performed in house for caspase-3 (rabbit-anti-cleaved-caspase-3 antibody,
retrieved by 20 min boiling in sodium citrate 10 mM pH 6.0/0.05% Tween-20. Endogenous peroxidases were inactivated with 0.3% hydrogen peroxide during 30 min. Primary antibodies were detected with the appropriate secondary antibodies, coupled to biotin (1:500, Jackson Immunoresearch, West Grove, PA, USA). Biotin was then complexed with streptavidin coupled to HRP (016-030-084, Jackson Immunoresearch). HRP activity was detected with the chromogenic substrate Novared (SK4800, Vector Laboratories, Burlingame, CA, USA). Sections were then mounted in PBS–glycerol and photographed on an Axiophot microscope (Carl Zeiss, Zurich, Switzerland).

PGE2 measurements

A PGE2 ELISA Kit (Abcam, Cambridge, MA, USA) was used to measure serum PGE2 levels in age-matched (9-month-old) control AdKO and celecoxib-treated AdKO mice, as per the manufacturer’s instructions.

Ki67 immunofluorescence

Paraffin-embedded sections underwent deparaffinization by Histoclear and a graded series of ethanol washes (100, 95, 75, and 50%), followed by antigen retrieval in Antigen Unmasking Solution, Citric Acid Based (Vector Laboratories), in a steamer for 20 min, followed by blocking of non-specific binding using 10% normal goat serum. Slides were incubated with rabbit-anti-ki67 (1:100; Thermo Fisher Scientific, Waltham, MA, USA) primary antibody at 4 °C overnight. Slides were washed three times with 0.1 PBS+0.01% Tween-20 buffer, then incubated with fluorochrome conjugated Alexa Fluor-555 goat anti-rabbit secondary antibody (1:400; Life Technologies) at room temperature for 1 h. Slides were mounted with cover slips using ProLong Gold Antifade Mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) mounting solution. Slides were examined for Ki67 staining using the Leica DMRX microscope equipped with fluorescence capabilities. Images were acquired with the DP72 camera (Olympus) using the CellSans Software (Olympus).
TUNEL assay

Cell apoptotic activities were studied by DeadEnd Fluorometric TUNEL System (Promega). We used paraffin embedded adrenal tissue sections and followed the manufacturer’s protocol. Adrenals and staining were visualized using the Leica DMRX fluorescence microscope.

Quantitative real-time PCR

For each sample, 1 μg of mRNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time PCR was performed in an Applied Biosystems 7500 Real-Time PCR System. mRNA expression levels were detected by caspase-1 (Casp1, Mm00438023_m1), interleukin 1 beta (Il1b, Mm00434228_m1), PTGS2 (COX2) (Ptgs2, Mm00478374_m1), sf1 (Nr5a1, Mm00446826_m1), β-catenin (Ctnnb1, Mm00483039_m1), cyclin D1 (Ccnd1, Mm00432359_m1), and cyp17a (Cyp17a1, Mm00484040_m1) TaqMan Gene Expression Assays (Applied Biosystems). Gene expression levels were normalized to Gapdh (TaqMan Rodent Gapdh control, Applied Biosystems). Gene expression in celecoxib-treated mouse adrenal glands was calculated relative to control AdKO adrenal glands using $2^{-\Delta\Delta Ct}$ values.

Statistical analysis

All analyses were performed using the two-tailed Student’s t-test. Differences at $P<0.05$ were considered statistically significant. Mean ± S.E.M. of at least in-triplicate experiments were calculated. Animals were age- and sex-matched for all comparisons between treated and untreated.

Results

Responses of human cell lines to celecoxib

Human adrenocortical cell lines CAR47 and H295R were treated with different concentrations of celecoxib. CAR47 is the first (and only) immortalized human cell line with an inactivating PRKAR1A mutation. It was derived from the adrenal gland of a patient diagnosed with PPNAAD and CNC (Nesterova et al. 2008). H295R is an adrenocortical carcinoma cell line that retains the ability to produce...
adrenal steroids (Rainey et al., 1994), including cortisol, whereas CAR47 does not (Rainey et al., 1994, Wang et al., 2012). HEK293 is a human embryonic kidney cell line (Graham et al., 1977) and was used here as a ‘control’ line against the immortalized adrenal cells of H295R and CAR47.

The three cell lines were treated with different concentrations of celecoxib for 24 h. Cell viabilities were then studied by MTT cell proliferation assay and the cell number was registered. Increased celecoxib concentrations led to decreasing cell viability. The CAR47 and H295R lines showed similar responses that were significantly different from HEK293 at 1, 3, 4, and 7 μM concentrations of celecoxib (Fig. 1A). Increasing celecoxib concentrations led to decreasing cell numbers with the CAR47 and H295R lines with fewer cells compared to HEK293 (Fig. 1B). In addition, celecoxib concentrations more than 2 μM reduced cortisol concentrations in H295R cell culture medium (Fig. 2A). However, if we normalize the decrease in cortisol concentrations by the number of cells, it appears that the decrease in cortisol levels was due to the effect of celecoxib on cell viability (Fig. 2B).

**Celecoxib treatment of AdKO mice: effects on the adrenal and corticosterone levels**

Following 6 months of celecoxib treatment, partial rescue of the phenotype caused by adrenocortical Prkar1a loss (Sahut-Barnola et al. 2010, de Joussineau et al. 2012) was observed: BAH of the adrenal cortex was seen in all 9-month-old AdKO mice that did not receive celecoxib, whereas none of the 9-month-old treated mice had clear evidence of BAH (Fig. 3). Moreover, plasma corticosterone levels in celecoxib-treated AdKO mice were significantly reduced compared to those in 9-month-old age-matched vehicle-treated AdKO mice after the 6-month-long treatment (Fig. 4A). This was accompanied by a decrease in serum PGE2 levels in the age-matched AdKO mice following the 6-month-long treatment (Fig. 4B).

Ki67 immunofluorescence was used for the assessment of cell proliferation of AdKO mouse adrenals (Fig. 5A), as previously described (Schluter et al. 1993). The number of Ki67-positive cells was reduced in the adrenal glands of celecoxib-treated mice compared to vehicle (Fig. 5B).

Celecoxib treatment rescued the resistance of AdKO adrenocortical cells to apoptosis, as measured by caspase-3 IHC (Fig. 6A) and TUNEL fluorescent assay (Fig. 6B), which were performed as previously described (Labat-Moleur et al. 1998). Apoptotic cells were detected only in the adrenal gland of celecoxib-treated mice, but not in those of untreated animals by both IHC and fluorescence (Fig. 6A and B).

**Changes in gene expression after celecoxib treatment**

RT-PCR analysis showed significant effects of 6 months of dietary celecoxib treatment on a number of genes. We found decreased expression of Casp1, Il1b, COX2 (Ptgs2), SF1 (Nr5a1), β-catenin (Ctnnb1), and cyclin D1 (Cnd1) in the adrenals of celecoxib-treated mice (Fig. 7A). We also analyzed the expression of different enzymes involved in the steroidogenesis. We found decreased gene expression of cyp11a and cyp17a, two enzymes involved in steroidogenesis in celecoxib-treated AdKO mice (Fig. 7B) compared with age-matched AdKO control, although only cyp17a expression was statistically significantly reduced. Thus, celecoxib treatment corrects at least in part the
overexpression of the Cyp17a enzyme that was previously observed (Sahut-Barnola et al. 2010) in AdKO mice.

Investigation of the GR in treated and untreated mice

Celecoxib has been shown to enhance the function of the GR (Hu et al. 2005) and GR has been implicated in human (Bourdeau et al. 2003) but not mouse (Louiset et al. 2009) BAH. We studied GR expression at the mRNA and protein level in AdKO mice both at baseline and after treatment with celecoxib (Fig. 8). AdKO mice showed no paradoxical increase in glucocorticoid secretion (data not shown) and there were no differences at the mRNA or at the protein levels by immunoblotting at baseline (Fig. 8A and B). Immunolocalization showed that GR was not even present in AdKO cortical steroidogenic cells and was mainly detected in non-steroidogenic cells (capsule, blood vessels and, as expected, in chromaffin cells; Fig. 8C). When we compared total GR expression in celecoxib-treated and vehicle, there were also no differences (Fig. 8D).

Figure 5
Effect of celecoxib on adrenocortical cell proliferation: (A) Ki67 staining in adrenals from mice untreated (control) and treated (celecoxib). Ki67 positive staining (red) and nuclear staining (blue). (B) As shown by quantification of Ki67-positive cells, 6 months of celecoxib treatment led to decreased proliferation in the adrenal cortex of AdKO mice.

Figure 6
Celecoxib treatment triggered apoptotic activity in the adrenal cortex of AdKO mice: (A) caspase-3 staining: no staining seen in the adrenal cortex of AdKO mice that received no treatment at the age of 9 months, whereas in mice treated with celecoxib, there were several caspase-3-positive cells. (B) TUNEL assay. Likewise, TUNEL-positive cells were seen (red arrows) only in the adrenal glands of celecoxib-treated animals, whereas no TUNEL-positive cells were seen in AdKO mice that were not treated with celecoxib.
Celecoxib, uniquely among all non-steroid anti-inflammatory drugs (NSAIDs), has been used extensively both in vitro and in vivo to inhibit neoplastic growth or proliferation (Bertagnolli 2006, Daikoku et al. 2014, Yokouchi et al. 2014, Kurtova et al. 2015). Some of the in vitro effects may be because at certain concentrations the drug is insoluble and, thus, causes precipitate-dependent effects and cellular toxicity (Sacchetti 2013). However, there are also true biologic effects of celecoxib, mostly linked to PGE2 inhibition and the consequent decrease of this molecule's induction of stem cell-like cells that are essential for the growth of at least some tumors (Kurtova et al. 2015).

We hypothesized that celecoxib might be useful in the treatment of PPNAD, because in both animals (Sahut-Barnola et al. 2010, de Joussineau et al. 2012) and humans with various forms of BAH (Carney et al. 2010, 2011, 2012, Almeida & Stratakis 2011), the disease has been linked to FLACs. In addition, in at least two other lesions caused by Prkar1a deficiency, mouse osteochondromyxomas and FDL tumors, PGE2 levels were elevated both at the tumor level and systemically, as well as linked to abnormal growth and proliferation of aBSCs, relatively undifferentiated cells of the osteoblastic lineage (Almeida et al. 2011). Indeed, our data support an effect of systemic celecoxib therapy in mouse BAH caused by Prkar1a deficiency, in addition to an inhibitory effect on human adrenal cell viability in culture.

COX2 (PTGS2) is expressed in the human and rodent adrenal cortex and cell lines, as previous studies have demonstrated (Salmenkivi et al. 2001, Engstrom et al. 2008, Martinez Calejman et al. 2011). Additionally, COX2 is activated in cells of the immune and/or inflammation system embedded within the cortex (Cover et al. 2001, Ichitani et al. 2001). In turn, corticosteroids suppress COX2 expression (Zhang et al. 1999). The present study suggested that celecoxib inhibition of COX2 led to decreased PGE2 levels and glucocorticoid secretion, but more importantly an improvement in the histologic phenotype of the BAH associated with Prkar1a deficiency. It is tempting to speculate that the decreased proliferation and increased apoptosis within the mouse adrenals affected by BAH and treated with celecoxib were due to an effect on the primary cells responsible for the phenotype: Prkar1a-null cells that are FLACs, whose continued proliferation in adult adrenal glands cause hyperplasia and tumors (Sahut-Barnola et al. 2010, de Joussineau et al. 2012). PGE2 is essential for maintaining stem cell-like cells in a number of tissues (Cha et al. 2006) and inhibiting the enzyme that degrades enhances regenerative capacity (Essex et al. 2013).

Our data support a possible role of celecoxib in the treatment of patients with PPNAD. However, human studies have not been performed. Celecoxib is widely used and there are a number of known side effects, but adrenocortical insufficiency is not one of them (Zhang et al. 2015). The latter may be because our data do not support an effect of celecoxib on normal adrenal cortex, but only in these glands where there is hyperplasia and/or FLACs.

The observed effects were not due to differences in GR expression in celecoxib-treated animals (Fig. 8). Celecoxib has been shown to enhance the function of the GR (Hu et al. 2005) and GR has been implicated in human (Bourdeau et al. 2003, Briassoulis et al. 2011) but not...
mouse (Louiset et al. 2009) forms of BAH. Humans with PRKARIA mutations and BAH (mostly PPNAD) demonstrate paradoxical increase in their glucocorticoid secretion in response to dexamethasone (Stratakis et al. 1999). However, this has not been seen in any of the animal models of PPNAD studied to date (Griffin et al. 2004a,b, Louiset et al. 2009, Sahut-Barnola et al. 2010) and it is not universally present among patients with BAH and PRKARIA mutations (Stratakis & Kirschner 1998, Stratakis et al. 1999, Hofland et al. 2012). GR expression was not increased in AdKO mice, the GR was barely present in adrenocortical steroidogenic cells, and its expression was not modified by celecoxib (Fig. 8).

In conclusion, our study of celecoxib, a COX2 inhibitor, showed promising results in the treatment of mice with a form of BAH caused by Prkar1a deficiency. Follow-up studies are needed but it is probable that NSAIDs that decrease PGE2 levels may provide a useful alternative to steroidogenesis inhibitors for patients with PPNAD.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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