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Prostaglandin receptor EP_1-mediated differential degradation of cyclooxygenases involves a specific lysine residue

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ABSTRACT

The cyclooxygenase (COX) enzyme isoforms COX-1 and COX-2 catalyze the main step in the generation of prostanoids that mediate major physiological functions. Whereas COX-1 is a ubiquitously expressed stable protein, COX-2 is transiently upregulated in many pathologies and is often associated with a poor prognostic outcome. We have recently shown that an interaction of COX-2 with the prostaglandin EP_1 receptor accelerates its degradation via a mechanism that augments its level of ubiquitination. Here we show that the sensitivity of both COX-1 and COX-2 to EP_1 is altered upon modification of one lysine residue. A point mutation of lysine to-arginine in position 432 of COX-2 (K432R) yields an enzyme with decreased sensitivity to EP_1-mediated degradation. In contrast, insertion of a putative ubiquitination site into the corresponding position of COX-1 (H446K′) yields an enzyme with higher levels of ubiquitination and reduced expression. Furthermore, compared to wild type COX-1, H446K′ is significantly more sensitive to downregulation by EP_1. Together these data suggest that distinctive ubiquitination of COX-1 and COX-2 may be responsible for their different sensitivity to EP_1-mediated degradation.

1. Introduction

The cyclooxygenase (COX) enzyme isoforms COX-1 and COX-2 catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins that play central roles in cardiovascular, immunological and brain function [1]. Both isoforms share a high degree of structural and catalytic similarities but differ profoundly in their stability [2]. Whereas COX-1 is a constitutively expressed and relatively stable protein ($t_{1/2} > 24$ h), COX-2 is short-lived ($t_{1/2} \sim 2$–5 h), and its expression is upregulated in many pathologies [3–6]. These differences have led to the formation of the COX-2 hypothesis whereby the ubiquitously expressed COX-1 is responsible for homeostatic functions, while the inducible COX-2 plays a predominant role in pathophysiological conditions (reviewed in [1,7]).

Evidences that accumulated over several years suggest that in addition to its catalytic function COX-2 also fulfills non-enzymatic roles in the cell, and therefore regulation of its levels may be as important as inhibiting enzymatic activity. For example, COX-2 was found to directly interact with the tumor suppressor p53 in the nucleus and thereby inhibit it's activity through a mechanism that does not involve enzymatic activity [8]. Interestingly, while the signaling cascades that lead to the induction of COX-2 are well-characterized [2], the cellular pathways that lead to degradation of COX-1 and COX-2 are partially characterized. Besides the differences in their stability the two isoforms differ in their place of degradation. In the absence of its principal fatty acid substrate arachidonic acid, the mature *N*-glycosylated COX-2 is shuttled directly from the ER via the ER-associated degradation (ERAD) pathway to the cytosol where it is subsequently degraded by the proteasome [9,10]. Degradation of COX-2 in this pathway is preceded by it polyubiquitination [10–12], but the identity of the specific lysine residues that undergo ubiquitination is not known. In contract to COX-2, under the same conditions COX-1 does not degrade in the proteasome and the location and mechanism of its degradation are not known.

Specificity of protein degradation in the proteasome is determined by a large family of ubiquitin-protein E3 ligases that recognize different motifs in the substrates. This recognition is modulated by molecular chaperones that render the substrate susceptible for recognition by the ligases via modification or association with protein substrates [13]. Two proteins have recently emerged as possible molecular chaperons for COX-2 degradation. Caveolin-1 was shown to facilitate the ubiquitination and degradation of COX-2 through an interaction with Derlin-1 [14,15], and we have recently shown that degradation of COX-2 is accelerated following its interaction with the G-protein coupled receptor prostaglandin E_1 (EP_1) [11]. We showed that the mechanism by which

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EP_1 downregulates COX-2 does not require enzymatic activity of COX-2 nor does it involve classical signal transduction by the receptor. Instead, EP_1 mediates an increase in COX-2 ubiquitination, thereby enhancing its degradation in the proteasome [11]. Given these findings, the purpose of the present study was to test whether COX-1 and COX-2 differ with respect to their sensitivity to the presence of EP_1 and whether this difference is due to their specific ubiquitination.

2. Materials and methods

2.1. Cell culture and transfection

All transfections were done in HEK 293 cells using PolyJet (SignaGen Laboratories) as described previously [11].

2.2. cDNA constructs

oCOX-1 and hCOX-2 cDNA constructs were gift of Prof. WL Smith, University of Michigan. YFP-tagged constructs were generated as described [11]. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for single site alterations. In the H446K′ construct, in addition to the lysine mutation the following amino acids were replaced: I440V, I444V, L445Q, H446K and K453E. All constructs were confirmed by sequence analysis.

2.3. Immunoprecipitation and immunoblotting

Monolayers in 100-mm culture dishes were washed twice with ice-cold PBS and lysed in RIPA/SDS buffer under denaturing conditions as described previously [11].

2.4. Radioimmunoassay

140,000 cells were plated per well in 12-well dishes and transfected as above, with each experimental point performed in triplicates. Prostaglandin E_2 (PGE_2) levels were measured by single antibody radioimmunoassay with dextran-coated charcoal precipitation as previously described [11].

2.5. Flow cytometry

Cells were washed twice with PBS, trypsinized and resuspended in 150–200 μl MEM for cytometric analysis. The samples were analyzed using BD FACSCanto II flow cytometer with DACSDiva software (BD Biosciences, San Jose, CA), as described previously [11].

2.6. Statistical analysis

All bars represent mean + SEM. Statistical analyses were done using the GraphPad Prism Software. Unless otherwise stated, statistical significance was determined by one-way ANOVA. p values <0.05 were considered significant.

3. Results and discussion

To assess the sensitivity of COX-1 and COX-2 to EP_1-mediated downregulation of expression we expressed either COX-1 or COX-2 in HEK 293 cells, in the absence or presence of EP_1. In each case, the effect of EP_1 on COX expression or activity was compared to its levels in the absence of the receptor. Measurements of enzymatic activity by conversion of arachidonic acid to PGE_2 revealed that co-expression of COX-2 with EP_1 caused a marked reduction in the activity of the enzyme (60%), while co-expression of COX-1 with EP_1 under the same conditions did not cause a significant change in enzymatic activity (Fig. 1A). A Western blot analysis confirmed that the changes in catalytic activity parallel changes in the level of protein expression (Fig 1B). Thus, while EP_1 significantly reduces the expression of COX-2, under the same experimental conditions its affect on the expression of COX-1 is significantly smaller.

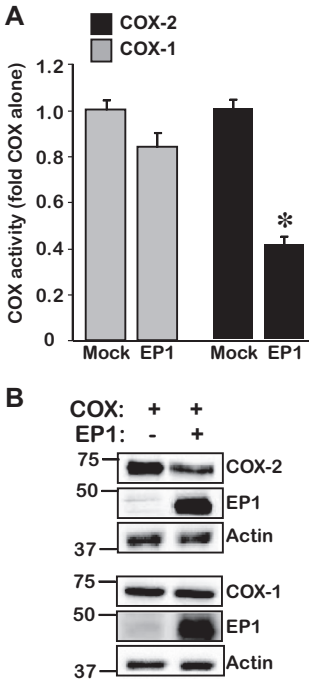

Fig. 1. COX-1 and COX-2 differ in their sensitivity to EP_1-mediated downregulation. (A) Cells transfected with 0.15 μg COX-2 or COX-1 and 0.75 μg of either Mock or EP_1 were incubated with 50 μM arachidonic acid for 30 min and enzymatic activity was measured by radioimmunoassay in $N = 4$ independent experiments performed in triplicates. $^*p < 0.05$ vs. empty vector. (B) Western blots of cell lysates from cells transfected with COX-2 or COX-1 in the absence or presence of EP_1 under the above transfection conditions.

Our previous study showed that the total ubiquitination content of COX-2 is elevated in the presence of EP_1 [11]. To begin to identify putative ubiquitination sites on COX-2 that may be involved in this process, we used the open access ubiquitination prediction software BDM-PUB [16]. Sixteen lysines were identified by the program as possible targets of ubiquitination at different probabilities. Ten of them had corresponding residues on COX-1, while six were unique to COX-2 (lysines 64, 68, 229, 344, 432 and 575). Lysines 575 and 432 received the highest score for likelihood of ubiquitination. However, since lysine 575 was within a 19 amino acid sequence that we previously found not to be involved in the EP_1-mediated downregulation of COX-2 [11], we chose lysine 432 as a candidate mediator for EP_1 unique regulation of COX-2 and tested the consequences of mutating this site. A lysine-to-arginine substitution in position 432 of COX-2 (herein K432R) caused only a mild reduction in the overall ubiquitination levels of COX-2 (Fig. 2A). Furthermore, as depicted both by Western blot and flow cytometry, this mutation did not affect the stability of the protein, as its expression was similar to that of the wild type COX-2 (Fig. 2B and C). Exposure of wild type and K432R COX-2 to the proteasome inhibitor MG132 caused accumulation of both (Fig. 2D), suggesting that substitution of a lysine in position 432 did not affect the degradation pathway of the protein.

We next sought to determine if deletion of the putative ubiquitination site on COX-2 alters its sensitivity to the presence of the EP_1 receptor. For this, we expressed YFP-tagged wild type COX-2 or YFP-K432R at constant levels, together with increasing concentrations of CFP-tagged EP_1, and measured receptor and COX-2

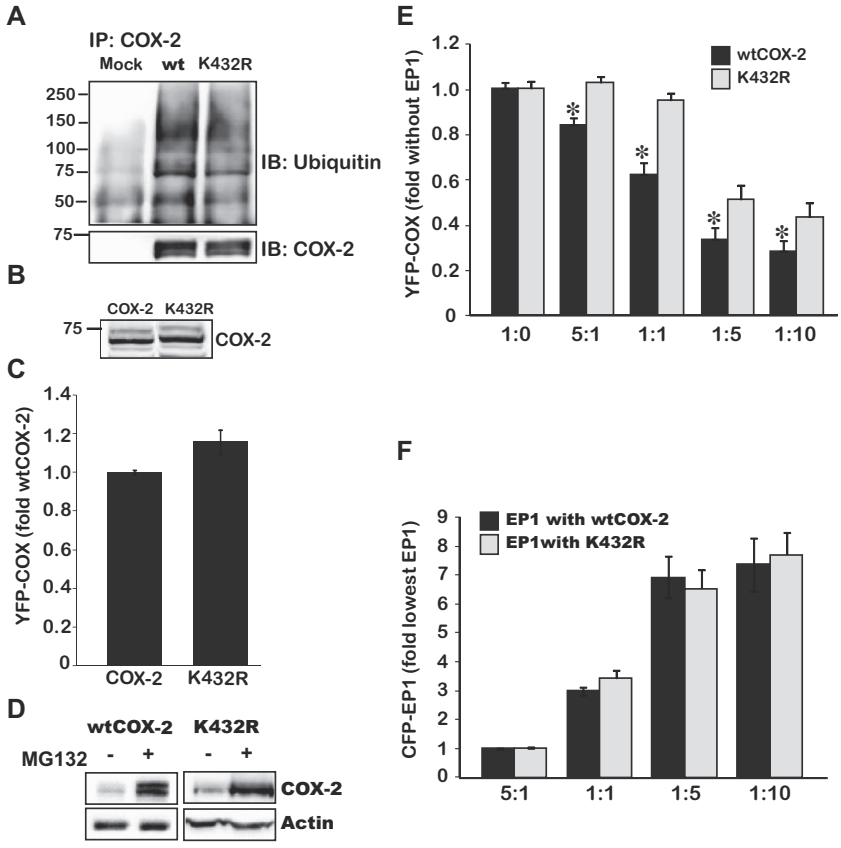

Fig. 2. (A) Lysine deletion in position 432 of COX-2 decreases its sensitivity to EP_1 mediated degradation. (A) HEK 293 cells transfected with 5 μg wild type or K432R COX-2 were subject to immunoprecipitation with anti-COX-2. Samples were separated and probed first with an anti-ubiquitin antibody (anti-mouse) followed by anti-COX-2 antibody (anti-goat). Shown is a representative of $N = 5$ independent experiments. (B) Representative Western blot depicting the expression of wild type and K432R COX-2. (C) Expression of YFP-tagged wtCOX-2 or YFP-K432R COX-2 using flow cytometry. (D) Cells expressing either COX-2 or K432R COX-2 were incubated with or without 20 μM MG132 for 8 h and tested for level of COX-2 (upper panel) and actin (lower panel). (E and F) HEK 293 cells were transfected with a constant amount of either wild type YFP-COX-2 or YFP K432R COX-2 and increasing levels of CFP-EP_1 at the ratios indicated. Expression was measured by flow cytometry. (E) Expression of YFP-wt COX-2 (black bars) or YFP-K432R (grey bars). (F) Expression of CFP-EP_1 in cells transfected with wtCOX-2 or YFP-K432R. The amount of total cDNA was kept constant using empty vector plasmid. $N = 5$ $^*p < 0.05$ wtCOX-2 vs. K432R.

expression using flow cytometry. The data presented in Fig. 2E revealed that the absence of a putative ubiquitination site on COX-2 had a significant effect on the ability of EP_1 to lower COX-2 expression. In accordance with our previously published data [11], a gradual increase in receptor expression caused a parallel decrease in the expression of wild type COX-2, even at relatively low levels of EP_1 (Fig. 2E, black bars). In contrast, K432R was not sensitive at all to EP_1 at the lower levels of receptor expression, and continued to be less effectively downregulated even at the highest levels of EP1 (Fig. 2E, grey bars). These data indicate that lysine 432 is involved in mediating the effect of EP_1 on COX-2. However, since it did not completely reverse the effect of EP_1, additional lysines are probably involved in the effect of EP_1 on COX-2 degradation. The expression of the EP_1 receptor was similar in the wild type and K432R expressing cells (Fig. 2F), supporting our earlier findings that the effect of EP_1 on COX-2 is unidirectional and COX-2 levels does not affect the expression of the receptor.

To further strengthen the concept that specific ubiquitination of COXs determines their sensitivity to EP_1, we tested whether introducing a lysine in the equivalent position of COX-1 (H446) can increase its sensitivity to EP_1. Despite the lack of clear consensus sequences of ubiquitination, Radivojac et al. found that putative ubiquitination sites show an abundance of negatively charged amino acids, depletion of hydrophobic residues, and absence of additional lysine sites immediately adjacent to the ubiquitination site [17]. We therefore designed a COX-1 mutant in which histidine in position 446 was substituted to lysine, and five amino acids neighboring the designated lysine were mutated as detailed in Section 2 (herein H446K′) to generate a favorable ubiquitination sequence. A comparison of expression using Western blot (Fig. 3A) showed that the expression of H446K′ was significantly lower than the wild type protein. We next tested whether insertion of the putative ubiquitination site into COX-1 affects its levels of ubiquitination. As shown in Fig. 3B, staining for ubiquitination shows similar levels between wild type and mutant COX-1. However, since the levels of H446K′ are significantly lower than those of COX-1, the amount of ubiquitinated protein is significantly higher in the mutant. Exposure of both wild type COX-1 and H446K′ to the proteasomal inhibitor MG132 resulted in a marked reduction in the expression of both (Fig. 3C). These results are in line with previous findings, which showed that in the absence of substrate COX-1 does not degrade in the proteasome [2]. Furthermore, as in the case of K432R COX-2, the insertion of a putative ubiquitination site into COX-1 did not change its degradation pathway.

To test whether the sensitivity of COX-1 H446K′ to EP_1 is affected by the mutation we expressed YFP-tagged wild type or mutant with two different expression levels of CFP-EP1. The results obtained in these experiments were supportive of those obtained with COX-2 and its ubiquitination-deficient mutant. Expression of wild type COX-1 with EP_1 at ratios of 1:5 and 1:10 caused a reduction in its expression, although the effect was significantly smaller than that of EP_1 on COX-2 at the same ratios (Fig. 2E). In contrast, H446K′ was significantly more sensitive to the presence of the EP_1 receptor compared to the wild type at both ratio of

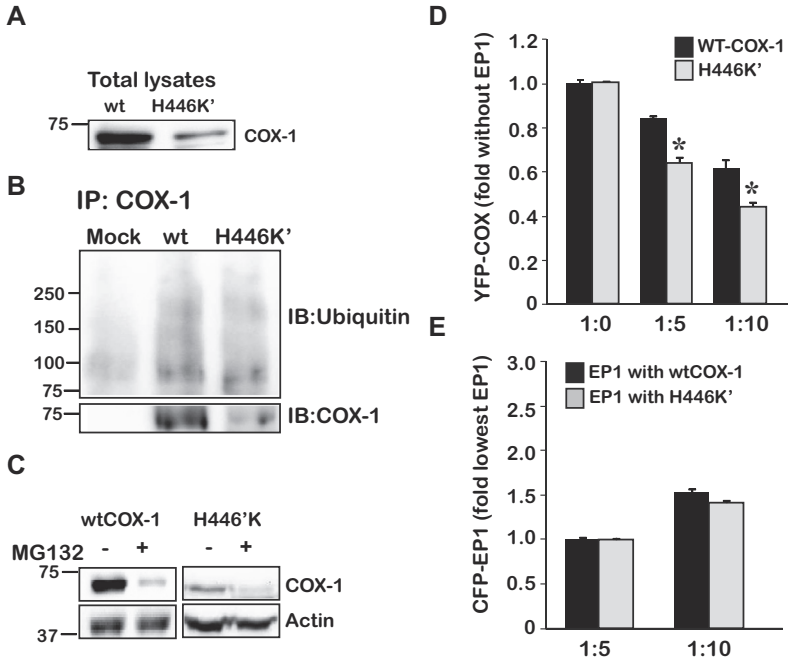

Fig. 3. Insertion of a putative ubiquitination site into COX-1 increases its sensitivity to EP_1. (A) Wild type COX-1 or H446K′ were expressed in HEK 293 cells for 16 h and expression levels were assessed by Western blot. (B) Cells were transfected with 5 μg DNA of empty vector (Mock), wild type COX-1 (wt) or H446K′ COX-1. Samples underwent immunoprecipitation with anti-COX-1 and probed with anti-ubiquitin antibody followed by anti-COX-1 antibody. Representative of $N = 4$, (C) Cells expressing either wild type COX-1 or H446K′ COX-1 were incubated with or without 20 μM MG132 for 8 h and tested for level of COX-1 (upper panel) and actin (lower panel). (D) HEK 293 cells expressing YFP-tagged COX-1 (black bars) or YFP-H446K′ COX-1 (grey bars) as function of increasing EP_1 DNA receptor levels. $^*p < 0.05$ wtCOX-1 vs. H446K′ $N = 5$, (E) CFP-EP_1 levels in the same cells as D. The amount of total cDNA was kept constant using empty vector plasmid.

transfection (Fig. 3D). As in the COX-2 experiments, the effect of the receptor on COX was found to be unidirectional since H446K′ did not affect the expression levels of the EP_1 receptor (Fig. 3E). Together these results suggest that insertion of a putative ubiquitination site into COX-1 increases its sensitivity to EP_1-mediated downregulation.

The degradation of COX-2 in the absence of arachidonic acid occurs via ERAD [7], followed by its ubiquitination by elements of the COP9 signalsome [12]. However, the identity of the sites that are ubiquitinated in the process is not known. The presence of several potential ubiquitination sites on COX-2 suggests that alternative patterns of ubiquitination may determine the pathway of degradation as well as modulate its kinetics. In the absence of EP_1, the K432R conversion did not affect the expression of general ubiquitination levels of COX-2 (Fig. 2) suggesting that K432 is not a primary site involved in COX-2 degradation in the above pathway. However, ubiquitination of K432 did affect the EP_1-mediated degradation of COX-2, suggesting that an interaction of COX-2 with chaperon proteins such as EP_1, may lead to alternative ubiquitination patterns that influence the levels of the protein. Interestingly, introduction of a putative ubiquitination site into COX-1 lowered its expression in the absence of EP_1 indicating that part of the differences in stability between COX-1 and COX-2 may be due to the absence of ubiquitination sites on COX-1. Future studies will determine whether the different ubiquitination patterns of COX-1 and COX-2 are affected by exposure to substrate, and by its interactions with other cellular proteins.

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