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Mini Review

Posttranscriptional and posttranslational determinants of cyclooxygenase expression

Uri R. Mbonye[1] *& Inseok Song*[2,*]

[1]Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106, USA, [2]Department of Life Science, University of Seoul, Seoul 130-743, Korea

Cyclooxygenases (COX-1 and COX-2) are ER-resident proteins that catalyze the committed step in prostanoid synthesis. COX-1 is constitutively expressed in many mammalian cells, whereas COX-2 is usually expressed inducibly and transiently. Abnormal expression of COX-2 has been implicated in the pathogenesis of chronic inflammation and various cancers; therefore, it is subject to tight and complex regulation. Differences in regulation of the COX enzymes at the posttranscriptional and posttranslational levels also contribute significantly to their distinct patterns of expression. Rapid degradation of COX-2 mRNA has been attributed to AU-rich elements (AREs) at its 3′ UTR. Recently, microRNAs that can selectively repress COX-2 protein synthesis have been identified. The mature forms of these COX proteins are very similar in structure except that COX-2 has a unique 19-amino acid (19-aa) segment located near the C-terminus. This C-terminal 19-aa cassette plays an important role in mediation of the entry of COX-2 into the ER-associated degradation (ERAD) system, which transports ER proteins to the cytoplasm for degradation by the 26S proteasome. A second pathway for COX-2 protein degradation is initiated after the enzyme undergoes suicide inactivation following cyclooxygenase catalysis. Here, we discuss these molecular determinants of COX-2 expression in detail. [BMB reports 2009; 42(9): 552-560]

INTRODUCTION

Prostanoids are twenty carbon fatty acid derivatives of arachidonic acid that are synthesized in almost all mammalian tissues. They are lipid hormones that act locally in an autocrine or paracrine fashion through G-protein coupled receptors to induce various physiological and pathological responses (1-4). Prostanoids regulate important homeostatic functions such as activation of innate immunity in response to microbial infection, maintenance of normal cardiovascular function and regulation of female reproductive biology (1, 4-6). Prostanoids that are aberrantly synthesized due to abnormal expression of COX-2 have been implicated in various pathologies such as chronic inflammation, fever, angiogenesis, and tumorigenesis (7-11). A key step in prostanoid synthesis is catalyzed by the cyclooxygenase isozymes, COX-1 and COX-2, which are heme-containing ER membrane-bound enzymes that possess two sequential catalytic activities (1, 3, 12-14). COX-1 and COX-2 have cyclooxygenase activity that bis-oxygenates free arachidonic acid (AA) that has been released from the *sn*-2 position of membrane glycerophospholipids by the action of phospholipase A_2. This leads to formation of the hydroperoxide prostaglandin G_2 (PGG_2), which is reduced at the peroxidase active site of the COX enzymes to form prostaglandin H_2 (PGH_2). Overall, the step catalyzed by COX-1 and COX-2 is rate-limiting for prostanoid formation and also considered to be the committed step in the prostanoid biosynthetic pathway (1, 3, 12). Cell specific prostanoid synthases then catalyze the isomerization or reduction of PGH_2 to form various bioactive prostanoids.

COX-1 and COX-2 are ER-resident *N*-glycoproteins that are structural and functional homodimers and exhibit ~60% identity in their primary structure (15-18). They are also integral membrane proteins that are monotopically inserted into the lumenal face of the ER membrane so that the opening of the cyclooxygenase active site is in the lipid bilayer to allow the uptake of mobilized free AA (13-15, 18-21). Despite their close structural and functional similarities, the COX enzymes are encoded by different genes that are differentially regulated and lead to distinct expression patterns and biological functions. COX-1 is constitutively expressed in many mammalian tissues under resting conditions (1, 22-27). In contrast, COX-2 is selectively expressed in some tissues in an inducible and transient manner (22, 23, 28-30). It has long been thought that the distinct temporal and spatial profiles of COX-1 and COX-2 expression are important in imparting these isozymes with different biological functions. For example, platelet cells do not express COX-2, but constitutively express COX-1 and thromboxane synthase, and it is well known that thromboxane A_2 (TXA_2) is synthesized due to functional coupling between COX-1 and thromboxane synthase (31).

*Corresponding author. Tel: 82-2-2210-2380; Fax: 82-2-2210-2888; E-mail: issong@uos.ac.kr

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Platelet-synthesized TXA_2 acts in an autocrine fashion to induce platelet aggregation and in a paracrine fashion to contract vascular smooth muscle cells (6). The anti-thrombotic and vasodilatory activities of prostacylin (PGI_2) counteract the effects of TXA_2 to achieve a balance that is crucial for maintenance of vascular homeostasis (32). Vascular endothelial cells synthesize prostacyclin under normal physiological conditions and during stress (33-35). Very low basal levels of PGI_2 can be detected in resting endothelial cells due to COX-1/PGI synthase coupling (36). In these cells, mechanical stimuli such as shear stress and inflammatory stimuli such as LPS or TNFα result in coordinate induction of COX-2 expression and a dramatic increase in the levels of PGI_2 (34-36). Vascular smooth muscle cells, which express high basal levels of PGI synthase (37), have also been reported to generate PGI_2 under stress and/or inflammatory conditions that induce COX-2 expression (38). In general, prostanoids generated as a result of COX-1 activity play housekeeping roles, while those generated by COX-2 are important physiologically for the control of normal innate immune function and female reproductive biology and have also been implicated in the aforementioned pathologies.

The distinct patterns of COX-1 and COX-2 expression have been attributed to differences in regulation of the isoforms at the transcriptional, posttranscriptional, and posttranslational levels. The COX-1 gene promoter is TATA-less, CAAT-less, GC-rich, and contains multiple transcription start sites (39). These promoter features are usually characteristic of housekeeping genes that are constitutively expressed under basal conditions. The COX-1 promoter also contains three potential SP1 binding sites at -610, -111, and -89 relative to the ATG start codon. Reporter gene assays have demonstrated that the SP1 sites at -610 and -111 are functionally important in maintaining basal constitutive expression of COX-1 (25). Unlike COX-1, the COX-2 5′ UTR is replete with *cis*-acting regulatory elements, which suggests tight and complex regulation of the gene by numerous signaling pathways. Depending on the cell type and the stimulus, distinct combinations of *cis*-regulatory elements will be utilized to activate COX-2 transcription. Of these *cis* elements, those that have been found to play a regulatory role in COX-2 transcription include E-Box, cAMP response element (CRE), NFκB, AP-1, CAAT enhancer binding protein (C/EBP), SP1, serum response element (SRE), and peroxisome proliferator response element (PPRE) (1, 40-42).

Evidence is mounting to show that the control of COX activity is quite complex and not restricted to regulation of the COX genes at the transcriptional level. In this minireview, we will focus our discussion on molecular determinants of posttranscriptional and posttranslational regulation of COX expression and activity that have been identified to date.

Posttranscriptional regulation of COX-1 and COX-2

COX-2 mRNA has a very short half-life (23) due to the presence of multiple copies of the AUUUA motif within the 3′-UTR of COX-2 mRNA that are known to direct mRNA decay (40, 43, 44). Deletion of these *cis*-acting decay motifs from the 3′-UTR of COX-2 stabilizes the transcript (23, 40). Unlike COX-2, COX-1 mRNA is very stable and its 3′-UTR lacks these AU-rich elements (AREs) (23). Although some ARE binding proteins have been shown to interact with COX-2 AREs (45-47), ARE binding proteins responsible for initiating ARE-mediated decay of COX-2 mRNA have yet to be identified. Activation of the p38 MAPK pathway by pro-inflammatory stimuli has been implicated in stabilization of the COX-2 mRNA (48, 49). Stabilization of the COX-2 transcript by p38 MAPK signaling is inhibited by glucocorticoid dexamethasone and is believed to require a 123-nucleotide region within the 3′-UTR and immediately downstream of the termination codon, which has six ARE copies (49). It is not clear which ARE binding proteins become activated by p38 MAPK signaling to effect ARE-dependent COX-2 stabilization; however, various ARE binding proteins such as CUGBP2 and HuR have been shown to bind the COX-2 3′-UTR and stabilize the transcript. HuR-mediated COX-2 transcript stabilization has also been reported in colon cancer cells where COX-2 is aberrantly over-expressed (45). In contrast, CUGBP2-mediated COX-2 transcript stabilization is observed in epithelial cells undergoing apoptosis due to radiation exposure (46, 47).

The translational silencing of COX-1 has been reported in megakaryocytes. During the phorbol ester-induced differentiation of the megakaryocytic cell line, MEG-01, COX-1 mRNA is upregulated within a day of phorbol ester treatment, whereas an increase in COX-1 protein is not observed for several days (26). Duquette and Laneuville have reported a correlation between the occupancy of a putative 20-nucleotide *cis* element within the COX-1 3′-UTR by a protein complex and the inhibition of COX-1 protein synthesis (26). This complex of COX-1 mRNA binding proteins has yet to be characterized.

COX-2-derived prostaglandins are required for normal embryo implantation in the uterus (50). Recently, Chakrabarty *et al*. identified two miRNAs (miR-101a and miR-199a) that appear to control COX-2 expression in the mouse uterus during embryo implantation (51). MicroRNAs are genome-encoded small (~19-22 nt) non-coding RNAs that bind to the 3′ UTR of certain mRNAs, resulting in their translational silencing or degradation (52, 53). In their study, Chakrabarty *et al*. demonstrated that the spatial and temporal expression of miR-101a and miR-199a in the uterus overlaps with that of COX-2 mRNA and is inversely correlated with COX-2 protein levels. Moreover, ectopic expression of miR-101a or miR-199a in HeLa cells repressed endogenous COX-2 translation (51). However, the biological significance of the microRNA control of COX-2 expression in the uterus has yet to be determined.

A structural determinant of proteasomal degradation of COX-2

The COX isoforms are structurally similar and have the same

subcellular localization pattern (15-18, 21, 22). Nevertheless, protein turnover experiments have demonstrated that COX-2 is much more susceptible to degradation than COX-1 (22, 54). In murine NIH/3T3 fibroblasts, COX-2 has a half-life of ~2 h as a result of degradation by the cytosolic 26S proteasome under conditions in which COX-1 is very stable ($t_{1/2}$ >12 h) (22). In agreement with this observation, Rockwell *et al*. reported the accumulation of COX-2 in its native form and as polyubiquitin conjugates in HT4 neuronal-like cells treated with inhibitors or disruptors of proteasomal degradation (55). In contrast, COX-1 protein levels were unaffected by this treatment (55). These results suggest that COX-2 may be selectively regulated by the ubiquitin-proteasome pathway, which has been implicated in the proteolysis of intracellular proteins with short half-lives. Since COX-2 is an ER-resident integral membrane protein, its degradation by the 26S proteasome must involve removal of the enzyme from the ER lumen and transportation across the ER membrane to the cytosol.

The maturation of COX-1 and COX-2 in the ER lumen involves cleavage of the N-terminal signal sequence, *N*-glycosylation at multiple sites, disulfide bond formation, heme incorporation, membrane insertion, and dimerization (1, 13, 16, 17, 56). The primary structures of COX-1 and COX-2 are very similar except that COX-2 has a 19-amino acid (19-aa) insertion near its C-terminal end (Asn-594-Lys-612). This C-terminal insertion imparts COX-2 with a consensus *N*-glycosylation site at Asn-594 that has no counterpart in COX-1. As a result, COX-1 is *N*-glycosylated at three sites, while COX-2 is glycosylated at its first three *N*-glycosylation sites and variably glycosylated at Asn-594 (Fig. 1A) (56, 57). Studies that have been conducted to determine the molecular basis for the relatively rapid rate of protein degradation of COX-2 when compared with that of COX-1 have shown that the C-terminal 19-aa segment is required for the proteasomal degradation of COX-2. Point mutation of the Asn-594 glycosylation site at the beginning of the insertion also stabilizes COX-2; however, the Asn-594 glycosylation site by itself is not sufficient to enable COX-2 protein degradation (Fig. 1B) (22). Interestingly, a COX-1 mutant possessing the COX-2 19-aa insertion near its C-terminus is constitutively glycosylated at Asn-594 and is also degraded in a proteasome-dependent manner (22, 54). These C-terminal mutations do not change the catalytic activities or the subcellular localization patterns of the enzymes (22).

ER-associated degradation of COX-2

The process by which ER-associated *N*-glycoproteins are selectively removed from the ER for degradation by the 26S proteasome has been termed ER-associated degradation (ERAD) (58-61). Experimental data from the aforementioned studies suggest that an *N*-glycosylated COX-2 C-terminal 19-aa insertion is critical for initiation of the entry of the protein into the ERAD pathway (22, 42, 54). These observations are similar to those of Spear and Ng, who found that a specific *N*-linked oligosaccharide in a multiple *N*-glycosylated glycoprotein can serve as the determinant for initiation of the ERAD of the protein (62). ERAD acts as a quality control pathway for the clearance of misfolded or structurally damaged proteins from the ER (58-61, 63). Very few native ER-associated integral membrane proteins other than COX-2 have been identified as ERAD substrates. These proteins include hydroxymethylglutaryl-CoA reductase, hepatic microsomal cytochrome P450 CYP3A4, and inositol 1,4,5-triphosphate receptor (64-66). COX-2 is a unique ERAD substrate because it is degraded from the native state and its topological features of being a wholly luminal integral membrane protein necessitate that the protein be transported out of the ER. ERAD glycoprotein substrates that are wholly luminal or membrane-bound with substantially large luminal domains are usually selected for degradation in the ER lumen (60). This is a complex process that involves the

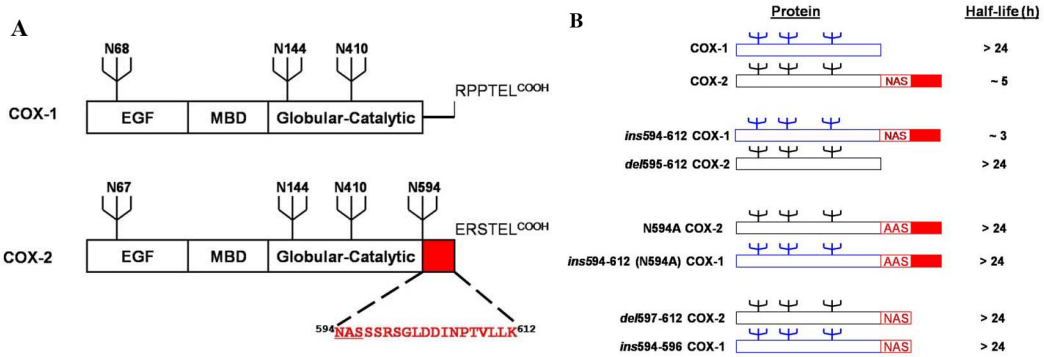

Fig. 1. The COX-2 C-terminal 19-aa is a protein instability element. (A) Domain alignment of the mature forms of COX-1 and COX-2. COX-1 can be glycosylated at three sites whereas COX-2 has four functional *N*-glycosylation sites. The last glycosylation site of COX-2 (Asn-594) is variably glycosylated and is part of a unique C-terminal 19-aa insertion (19-aa) whose sequence is shown. EGF, epidermal growth factor-like domain; MBD, membrane binding domain. (B) Stability of COX C-terminal 19-aa mutants heterologously expressed in HEK293 cells.

enzymatic processing of specific *N*-glycan moieties on the surface of the glycoprotein and requires the action of α1,2 ER mannosidase I (67-70). Selective inhibition of α1,2 ER mannosidase I activity by kifunensine stabilizes COX-2 and other glycoproteins by preventing their removal from the ER lumen (22, 54, 67-71).

It has been proposed that the ERAD of a glycoprotein requires both an *N*-linked glycan component and a non-native protein structure (72). This model stems from the observation that misfolded proteins possess an *N*-glycan moiety; pharmacological or genetic inhibition of *N*-glycosylation causes the retention of misfolded proteins in the ER and prevents their degradation (72). The COX-2 C-terminal 19-aa insertion has not been successfully resolved in the X-ray crystal structure of COX-2, which may indicate that it is substantially lacking in secondary structure. The last resolved residue in the highest resolution murine COX-2 crystal structure attained is Ser-596, which is the final amino acid in the Asn-594 *N*-glycosylation consensus sequence at the beginning of the insertion (14, 18). This *N*-glycosylation site is part of a two-turn helix (Helix A) that is linked to an upstream helix (Helix B) by a long 15- residue loop that includes 11 resolved residues (KGCPFTSFNVQ) and an unresolved 4-residue sequence (DPQP) (Fig. 2). Asn-594 is not glycosylated in the murine structure, presumably because the amide group of its side chain is pointed upwards towards Helix B, which is ~4.5 Å away. This space is barely sufficient to accommodate an *N*-glycan group. Moreover, a disulfide bridge between Cys-569 of the upstream helix and Cys-575 of the loop may further hinder Asn-594 glycosylation. Therefore, for Asn-594 to be glycosylated, a local conformational change would have to occur. Such a change would likely involve breakage of the disulfide bond and movement of the long and inherently flexible intervening loop. The hydroxyl side chain of Ser-596 is solvent-exposed and situated near the surface of the membrane. The KDEL-like C-terminal ER retention signal of COX-2 also has to be solvent-exposed and near the membrane in order to be bound by membrane proteins involved in ER retention. Therefore, the unresolved portion of the COX-2 insert may occur on the surface of the protein close to the membrane surface.

Immunoblotting of a variety of cell lines for COX-2 usually reveals the presence of 72 and 74 kDa variably glycosylated

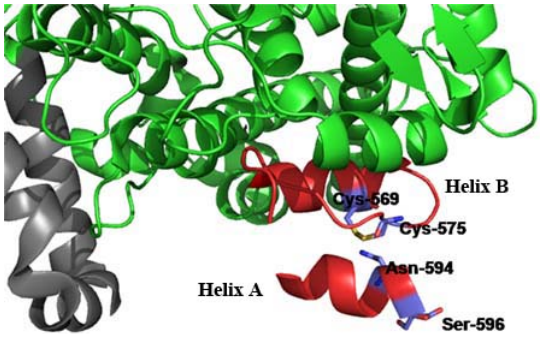

Fig. 2. A ribbon diagram highlighting the C-terminal structure of murine COX-2. The last resolved residue in this structure is Ser-596, which is the final amino acid of the Asn-594 consensus glycosylation site. Not surprisingly, Asn-594 is not glycosylated, probably because its amide side chain is pointed away from the surface and in the direction of a nearby helix. Both helices (Helix A and Helix B) are separated by a long 15-residue loop; the last four residues of this loop are not resolved in the structure. A disulfide bond is formed between the thiols of Cys-569 and Cys-575. The distance between Asn-594 and Cys-569 is not sufficient to accommodate an *N*-glycan group. For Asn-594 to become glycosylated there has to be a local conformational change in the C-terminal structure that would allow the amide group of Asn-594 to be exposed to the surface. The catalytic domain is colored green and the membrane binding domain is colored gray.

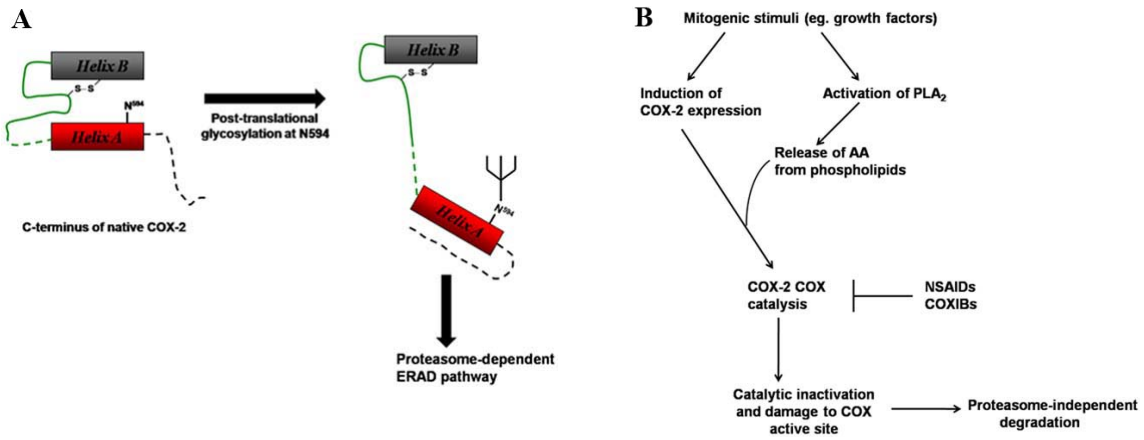

Fig. 3. Proposed pathways for COX-2 protein degradation. (A) Basal degradation of COX-2 is initiated by the C-terminal 19-aa insert. Posttranslational glycosylation at Asn-594 triggers the removal of COX-2 from the ER via the ERAD pathway for proteasomal degradation. (B) Substrate-dependent degradation of COX-2 proceeds from the suicide inactivated, structurally damaged enzyme.

forms of the enzyme due at least in part to alternative glycosylation at Asn-594 (56, 57). Inhibition of the co-translational *N*-glycosylation of COX-2 by tunicamycin eliminates cyclooxygenase and peroxidase activities of the enzyme (56). However, point mutation of the Asn-594 glycosylation site actually enhances the COX-2 COX specific activity (56), which likely occurs due to the substantially reduced turnover of the mutant enzyme (22). These results imply that, while the Asn-594 glycosylation site is not required for proper co-translational protein folding, it is essential for degradation of the enzyme. In a HEK293 heterologous system expressing recombinant native human COX-2, the Asn-594 glycosylated form of the protein accumulates with time upon addition of kifunensine (54). This is an indication that Asn-594 glycosylated COX-2 is rapidly degraded by ERAD. Mutations of the 19-aa insertion prevent *N*-glycosylation at Asn-594 and stabilize COX-2 (22, 54). Conversely, disruption of the helical conformation of Helix A improves the ease with which COX-2 is glycosylated at Asn-594 concomitant with enhancement of the overall extent of COX-2 protein degradation (54). It is also important to note that, unlike COX-2, COX-1 mutants possessing the COX-2 19-aa insertion lack secondary structure in the region upstream of the Asn-594 glycosylation site. As a result, COX-1 mutants are fully glycosylated at Asn-594 and degraded more rapidly ($t_{1/2} \sim 3$ h) and efficiently than native COX-2 ($t_{1/2} \sim 5$ h) in HEK293 cells (54). Overall, these observations have led to the model that the floppy region of the 19-aa insertion removes the impediment created by the Helix A-loop-Helix B region upon glycosylation at Asn-594. Posttranslational glycosylation of COX-2 can then trigger entry of the protein into the ERAD system.

Suicide inactivation of COX catalytic activity

Arachidonic acid is the endogenous fatty acid substrate of COX-1 and COX-2 (1, 12), which is mobilized from the *sn*-2 position of membrane phospholipids by the action of phospholipase A_2. At the cyclooxygenase active site, arachidonic acid is oxygenated to form the hydroperoxide and endoperoxide prostaglandin G_2 (1, 12, 73). This prostaglandin intermediate is then moved to the peroxidase (POX) site, where its hydroperoxy group undergoes a two-electron reduction to form the alcohol prostaglandin H_2 (1, 12, 73). PGH_2 is a common substrate for downstream terminal prostanoid synthases that act upon it differently to form various prostanoids. The COXs are the best known cellular target for non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit catalysis at the COX active site (1, 3).

The POX and COX activities of COX-1 and -2 undergo irreversible suicide inactivation *in vitro* during catalysis (1, 12). POX and COX self inactivation is mechanism-based because it proceeds from heme and tyrosyl radical intermediates that are formed during the POX and COX reactions, respectively (12, 74). However, the specific structural changes in the holoenzymes that lead to suicide inactivation remain unknown. Mevkh *et al*. noted that COX-1 self inactivation is accompanied by dramatic changes in protein structure as evidenced by the increased susceptibility of the inactive enzyme to trypsin cleavage and the increased number of exposed histidine residues subject to chemical covalent modification (75). If the COXs undergo suicide inactivation *in vivo* it could serve as an additional regulatory mechanism for prostanoid synthesis. In this regard, it would be interesting to determine if the inactive forms of COX-1 and -2 are more susceptible to degradation than the native forms because it is well known that structurally damaged proteins that are present in the ER are also degraded via the ERAD pathway.

Substrate-dependent degradation of COX-2

Recently, a second pathway for COX-2 degradation has been identified that is not proteasome-dependent and that appears to proceed from the suicide inactivated, structurally damaged form of the enzyme (Fig. 3) (54). This pathway was identified based on the observation that COX-2 protein degradation in serum-induced NIH/3T3 fibroblasts can be significantly retarded by NSAIDs, such as flurbiprofen and aspirin, as well as COX-2 selective inhibitors, such as NS-398 (21). Because NSAIDs do not inhibit the 26S proteasome, there must be at least two distinct pathways for COX-2 degradation in NIH/3T3 cells. Serum stimulation of NIH/3T3 cells results in the coordinate induction of COX-2 expression and the release of free AA due to activation of phospholipase A_2 (76). Therefore, it is reasonable to expect that serum-treatment of 3T3 cells will stimulate COX-2 COX catalysis. During catalysis, COX-2 may undergo suicide inactivation, which could initiate the rapid degradation of the enzyme. In contrast to NIH/3T3 cells, the degradation of heterologously expressed COX-2 in HEK293 cells is not significantly prevented by NSAIDs, which coincides with the fact that these cells lack detectable phospholipase A_2 activity (54, 77). The basal degradation of COX-2 in HEK293 cells can be substantially enhanced by the addition of exogenous AA at concentrations as low as 5 μM (54). Additionally, NSAIDs completely inhibit substrate-induced degradation of COX-2 in these cells without affecting its basal proteasome-dependent degradation (54). A COX inactive mutant, G533A COX-2, has a basal degradation half-life similar to the wild-type protein; however, this mutant is completely refractory to substrate dependent degradation (54). Overall, these findings suggest that a functional COX active site is required to mediate substrate dependent degradation of COX-2.

Upon exposure to exogenous AA, the catalytic activities of both COX-1 and COX-2 in intact HEK293 cells become inactivated (54). Therefore, the substrate dependent degradation of COX-2 may occur as a result of structural damage that causes catalytic inactivation of the enzyme. If this is the case, it is not clear why COX-1 is resistant to substrate dependent degradation even though it undergoes substrate-induced catalytic in-

activation (54). The pathway responsible for substrate dependent COX-2 degradation has yet to be fully characterized. We and others have found that this process occurs independently of the proteolytic activities of the 26S proteasome or the lysosome (54). Therefore, it is likely that the quality control of structurally defective ER-associated proteins could also be undertaken by a proteolytic pathway distinct from the classical ERAD-proteasome pathway.

Conclusion and perspectives

Prolonged synthesis and expression of COX-2-derived prostanoids has been linked to the pathogenesis of chronic inflammation and cancer. Here, we discussed various studies that have been conducted to investigate the molecular basis for the physiological short-lived expression of COX-2 and its prostanoid products. At the transcript level, COX-2 expression is tightly regulated by 3′ UTR *cis*-acting AREs and *trans*-acting miRNAs that block protein translation and/or initiate rapid degradation of the mRNA. At the protein level, we and others have identified a C-terminal instability element that is critical for the proteasomal degradation of COX-2 via the ERAD system. A second distinct mechanism for COX-2 degradation is preceded by the COX substrate-induced suicide inactivation of the enzyme. Substrate-dependent degradation could complement the basal proteasomal degradation of COX-2 in mammalian tissues that co-express significant PLA_2 activity. Since the differential expression profiles of COX-1 and COX-2 are thought to contribute to their specialized biological functions, it would be important to document the phenotype created by replacing the COX-2 endogenous gene with a degradation-resistant COX-2 mutant.

ERAD is an important protein quality control pathway that is not well characterized in mammalian cells. ERAD components in the ER lumen that participate in the initial selective recognition of an ERAD substrate and the exact mechanisms by which they do so are not clearly defined. The identity of the ER membrane retrotranslocon that exports proteins to the cytoplasm for proteasomal degradation has also yet to be determined. Therefore, since COX-2 is one of very few mammalian proteins that are known to undergo proteasomal degradation from the ER it may serve as a suitable reporter substrate for the evaluation of ERAD in a mammalian system.

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