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GLYCOSYLATION OF HUMAN CYCLOOXYGENASE-2 (COX-2) DECREASES THE EFFICACY OF CERTAIN COX-2 INHIBITORS

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Abstract

Prostanoids play an important role in a variety of physiological and pathophysiological processes including inflammation and cancer. The rate-limiting step in the prostanoid biosynthesis pathway is catalyzed by cyclooxygenase-2 (COX-2). COX-2 exists as two glycoforms, 72 and 74 kDa, the latter resulting from an additional glycosylation at Asn^{580}. In this study, Asn^{580} was mutated, and the mutant and wild-type COX-2 genes were expressed in COS-1 cells to determine how glycosylation affects the inhibition of COX-2 activity by aspirin, flurbiprofen, ibuprofen, celecoxib, and etoricoxib. Results indicate that certain inhibitors were 2- 5 times more effective at inhibiting COX-2 activity when the glycosylation site was eliminated, indicating that glycosylation of COX-2 at Asn^{580} decreases the efficacy of some inhibitors.

Keywords: cyclooxygenase-2 glycoforms; glycosylation; enzyme inhibition; NSAIDs; selective COX-2 inhibitors

Abbreviations: COX, cyclooxygenase; Asn, asparagine; AA, arachidonic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; kDa, kilodalton; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; PGE_2, prostaglandin E_2; NSAIDs, non-steroidal anti-inflammatory drugs; ER, endoplasmic reticulum

1. Introduction

Prostanoids, which consist primarily of prostaglandins, prostacyclins, and thromboxanes, are a family of lipid-soluble, bioactive compounds that can trigger various physiological responses and processes such as platelet aggregation, bone metabolism, ovulation, smooth muscle contraction and relaxation, neuronal plasticity, vascular permeability, and inflammation [1,2]. The rate-limiting step in the formation of these compounds is catalyzed by the enzyme cyclooxygenase (COX), also known as

prostaglandin H_2 synthase [3,4], which is an integral membrane protein located in the ER and nuclear envelope [5,6]. COX carries out two distinct reactions. The first is a cyclooxygenase reaction wherein arachidonic acid (AA)—an omega-6 fatty acid— is converted to prostaglandin G_2 (PGG_2); and the second is a peroxidase reaction in which PGG_2 is converted to prostaglandin H_2 (PGH_2) [7]. PGH_2 is then quickly converted to various active prostaglandins, prostacyclins, and/or thromboxanes by downstream synthases.

Two true isoforms of COX have been found: the constitutively expressed "housekeeping" enzyme COX-1 [8-12], and COX-2, which— depending on the tissue— can be inducible or constitutive [1,13-15]. While COX-2 is generally perceived to be involved primarily in pathological conditions, several studies have found that this enzyme plays an essential role in various normal physiological processes as well, such as neurotransmission and synaptic activity [16,17], maintaining normal renal functions [18], regulating cerebral blood flow in newborns [19], facilitating pregnancy [20,21], T cell development [22], and ulcer healing [23]. However, despite the fact that normal COX-2 expression is necessary for maintaining health, the negative impacts that COX-2 expression can have on the body have generated much more attention in the scientific and medical communities. COX-2 was originally implicated as the main enzyme involved in the production of pro-inflammatory prostanoids [1]. Overexpression of COX-2 occurs in several cancers, most notably colon [24], breast [25], and prostate [26,27]. Other diseases in which COX-2 is believed to be associated are rheumatoid arthritis, osteoarthritis, and Alzheimer's disease [1,2]. This has led to the dissemination of various COX inhibitors known as non-steroidal anti-inflammatory drugs (NSAIDs) in an effort to either prevent or slow down the progress of these COX-2-induced diseases. Most traditional NSAIDs— such as aspirin, ibuprofen, naproxen, and flurbiprofen— are categorized as nonselective COX inhibitors, in that they inhibit both COX-1 and COX-2 activities. Unfortunately, the inhibition of the "housekeeping" COX-1 enzyme can lead to serious side effects, such as gastrointestinal complications, renal problems, and platelet

dysfunction [1]. In an effort to remedy this problem, researchers developed selective COX-2 inhibitors such as celecoxib (known commercially as Celebrex) and rofecoxib (known as Vioxx) [28]

An additional complexity is that COX-2— which is always N-glycosylated at three sites— exists as at least two distinct glycoforms due to an N-linked glycosylation site at Asn^{580} that is modified only ~50% of the time [29]. The unmodified and modified glycoforms have molecular weights of 72 and 74 kDa, respectively. Our lab determined that this glycosylation site plays an important role in enzyme turnover [30], and this finding has since been confirmed by other researchers [31]. Our previous study also revealed the existence of a third glycoform (70 kDa) which is expressed at high levels only when expression of the 74 kDa glycoform was blocked.

In this present study, we sought to determine if glycosylation at Asn^{580} has any influence on the inhibitory action of some of the more frequently used COX-2 inhibitors, namely aspirin, ibuprofen, flurbiprofen, celecoxib, and etoricoxib. Our findings suggest that this additional glycosylation does indeed impede the action of traditional NSAIDs like aspirin, ibuprofen, and flurbiprofen and the selective inhibitor etoricoxib but appears to have no significant impact on the action of the selective inhibitor celecoxib.

2. Materials and methods

2.1 Materials

The human COX-2 cDNA in plasmid pcDNA3 was generously provided by Dr. Timothy Hla from the Weill Cornell Medical College, USA. The Asn^{580}-mutant COX-2 gene (wherein asparagine was replaced with a glutamine residue) was created as described previously [30]. The COS-1 cell line was obtained from the UCSF Cell Culture Facility (San Francisco, CA, USA). QIAprep Spin Miniprep kit and the HiSpeed Plasmid Maxi kit were purchased from QIAGEN (Valencia, CA, USA). One Shot TOP10 Competent *E. coli* cells were obtained from Invitrogen (Carlsbad, CA, USA). The transfection reagent *Trans*-IT LT1 was purchased from Mirus Bio (Madison, WI, USA). The anti-human COX-2

polyclonal antibody, the Prostaglandin E_2 EIA kit—Monoclonal, peroxide-free arachidonic acid (AA), and ibuprofen were purchased from Cayman Chemical (Ann Arbor, MI, USA). Aspirin and flurbiprofen were purchased from Sigma (St. Louis, MO, USA). Celecoxib was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Etoricoxib was purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Transfection of COS-1 cells

One Shot TOP10 Competent *E. coli* cells were used to produce large quantities of plasmid containing either the wild-type or mutant COX-2 gene. Plasmids were isolated and purified using the HiSpeed Plasmid Maxi kit according to the manufacturer's instructions. COS-1 cells were grown on 6-well plates in DMEM, 5% FBS media at 37°C. *Trans*-It LT1 reagent was used to transiently transfect cells with either the wild-type or mutant COX-2 gene according to the manufacturer's instructions. Cells were incubated at 37°C for ~48 hours in the presence of the *Trans*-It LT1 /COX-2 DNA complex. Media was then replaced with DMEM, 1% FBS, 4 mM L-glutamine, and antibiotics, and the cells continued their incubation.

2.3. Treatment of COX-2 expressing cells with COX-2 inhibitors and analysis of COX-2 activity

Approximately three days after transfection, groups of COS-1 cells were treated with various concentrations of aspirin, ibuprofen, flurbiprofen, celecoxib, or etoricoxib for 1 hour at 37 °C. All cells were subsequently treated with 5 µg/ml of AA for 2 hours at 37 °C, as previously described [30]. After the inhibitor and AA treatments, media samples were collected and analyzed for the presence of the downstream product prostaglandin E_2 using a Prostaglandin E_2 EIA kit—Monoclonal according to the manufacturer's instructions.

2.4. Western blot analyses

After media removal, COS-1 cells growing on 6-well plates were washed with ice-cold PBS and then lysed in 50mM MOPS buffer containing 5mM EDTA, 10mM EGTA, 1% Triton X-100, and a protease inhibitor cocktail. The whole cell lysates were sonicated briefly and centrifuged at 14,000x g for 5 minutes to remove cytoskeletal structures. Protein concentrations were determined and separated using a 7% SDS-polyacrylamide gel. COX-2 expression was monitored with Western blotting using α-COX-2 antibody and images were captured digitally (Carestream Image Station 4000MM PRO). β-actin is a housekeeping protein used to ensure equal protein loading.

3. Results and discussion

3.1. Effect of glycosylation at Asn^{580} on the efficacy of COX inhibitors

In a previously published study [30], our lab showed that COS-1 cells do not express endogenous COX-2 protein and therefore are a suitable background for expressing our COX-2 constructs. Transfection of the COS-1 cells with the wild-type COX-2 gene resulted in expression of two COX-2 glycoforms with molecular weights of 72 and 74 kDa [30]. In that same study, we demonstrated that the elimination of the Asn^{580} site in the COX-2 protein— which led to the elimination of the 74 kDa glycoform— affected COX-2 turnover, resulting in a significant increase in total COX-2 activity, a build-up of the 72 kDa form, and the appearance of a new 70 kDa glycoform. Shortly thereafter, another group also reported the expression of this 70 kDa glycoform in COX-2-expressing cells treated with glucosamine hydrochloride [32].

The fact that total COX-2 protein levels increase substantially when the Asn^{580}- glycosylation site is eliminated can unfortunately complicate comparisons made between the total COX-2 activity expressed from the wild-type COX-2 gene and that expressed from the Asn^{580}-mutant COX-2 gene. In this present study, the effect of COX-2 glycosylation on inhibitor efficacy was analyzed; however, standard IC_{50}

comparisons could not be made since IC_{50} values are significantly affected by enzyme levels. Therefore, in order to determine if glycosylation of COX-2 influences the effectiveness of COX-2 inhibitors— irrespective of total COX-2 protein levels— another analytical approach was needed. Initially, all inhibitors were tested over a broad range of concentrations (Figure 1). Analyzing these data then allowed us to choose a narrower range of concentrations that would facilitate a simple linear regression analysis for each inhibitor (Figure 2). As in our previous report, COX-2 activity was followed by measuring the more stable, downstream product PGE_2 [30].

Three nonselective COX inhibitors, or traditional NSAIDs, were chosen for this study— aspirin, flurbiprofen, and ibuprofen. In addition, the selective COX-2 inhibitors celecoxib and etoricoxib were also tested. The effect of aspirin on COX-2 activity is shown in Figures 1A and 2A. We determined that the concentration range of 2 to 10 μM aspirin allowed for a simple linear regression analysis of both the "wild-type" 72/74 and the "Asn^{580}-mutant" 70/72 kDa COX-2 activities (Fig. 2A). As Figure 2A shows, the slope of the "70/72 kDa" line (-209.25) is ~5-fold steeper than that of the "72/74 kDa" line (-40.87), indicating that aspirin is approximately five times more effective at decreasing the activities of the 70/72 kDa glycoforms than the 72/74 kDa glycoforms. The inhibitory action of flurbiprofen was best studied between 0.02 and 0.1 μM (Fig. 1B and 2B), and as Figure 2B demonstrates, the slope of the "70/72 kDa" line is ~4-fold steeper than that of the "72/74 kDa" line. Therefore, as with aspirin, flurbiprofen is apparently more effective at decreasing the activities of the 70/72 kDa enzymes than the 72/74 kDa enzymes. Figures 1C and 2C demonstrate the linear range for the inhibitor ibuprofen to be between 0.05 and 0.8 μM. Comparing the slopes of the 70/72 kDa activities versus the 72/74 kDa activities shows ~5-fold difference (Fig. 2C), again indicating that the 70/72 kDa glycoform activities are more susceptible to inhibition by NSAIDs. By contrast, the selective COX-2 inhibitor celecoxib appeared to inhibit the 70/72 and the 72/74 kDa activities almost equally well (Fig. 1D and 2D). The slopes for the two sets of COX-2 glycoforms (-154.63 for the 70/72 kDa and -113.27 for the 72/74 kDa glycoforms) were about equal when activities were measured over the linear range of 1.0 to 5.0 nM celecoxib (Fig. 2D). The linear range for

the selective inhibitor etoricoxib was 10-fold greater than that of celecoxib, ranging from 0 to 50 nM (Fig. 1E and 2E). Unlike celecoxib, etoricoxib was about 2 times more effective on the 70/72 kDa glycoforms than on the 72/74 kDa glycoforms (Fig. 2E)

3.2. Effect of COX inhibitors on COX-2 protein levels

To confirm that the COX-2 inhibition results shown above were not due to changes in COX-2 protein levels but rather due to changes in COX-2 activity caused by direct inhibitor binding, Western blotting was carried out on lysates prepared from COX-2-transfected COS-1 cells treated with various concentrations of aspirin, flurbiprofen, ibuprofen, celecoxib, and etoricoxib. Figure 3 shows the COX-2 glycoform profiles at various concentrations of two traditional NSAIDs (aspirin and flurbiprofen) and the two selective COX-2 inhibitors (celecoxib and etoricoxib). As inhibitor concentration increases, there appears to be no significant increase or decrease in either the 72/74 kDa glycoforms or the 70/72 kDa glycoforms. Similar results were seen with ibuprofen (data not shown).

3.3. Conclusion

Although the role of glycosylation and COX-2 enzyme turnover has been well-established [30,31,33], no other function has been attributed to this variable glycosylation site at Asn^{580}. It is, however, quite likely that glycosylation of COX-2 has other impacts on COX-2 function. Just as we accept the premise that the addition or subtraction of a relatively small phosphate group can alter the conformation and/or activity of a protein, we must also acknowledge that modification of a protein by a rather large carbohydrate moiety can have quite an impact on a protein's solubility, 3-D conformation, protein-protein interactions, and protein-inhibitor interactions [34].

In this current study, we chose to test the effect of COX-2 glycosylation on the efficacy of five COX inhibitors that represent the four inhibitor types outlined by Kurumbail et al. [35]. Aspirin— an irreversible inhibitor of both COX-1 and COX-2— is the only inhibitor known to covalently modify a

serine residue in the cyclooxygenase active site, thus preventing binding of the substrate arachidonic acid [36,37]. Ibuprofen is a classic, reversible, competitive inhibitor of both COX enzymes [36,38]. Flurbiprofen is a slow, time-dependent, irreversible competitive inhibitor of both enzymes [36,38]. Celecoxib and etoricoxib are time-dependent, slowly reversible inhibitors; however, unlike the other three compounds that interact solely in the cyclooxygenase site, these selective COX-2 inhibitors also interact with amino acids that reside in a "pocket" adjacent to the active site [35,39,40]. This site is not easily accessible in the COX-1 enzyme, and therefore, inhibitors such as celecoxib and etoricoxib form tighter complexes with COX-2 than with COX-1. Our results indicate that the addition of a carbohydrate group at Asn^{580} of the COX-2 enzyme decreases the efficacy of the traditional NSAIDs (i.e. aspirin, flurbiprofen, and ibuprofen) and the selective inhibitor etoricoxib but has no significant effect on the inhibitory action of the selective COX-2 inhibitor celecoxib. We speculate that small conformational changes occur in the active site when Asn^{580} is glycosylated, and this in turn reduces COX-2's affinity for most inhibitors. Why celecoxib binding is essentially unaffected by this glycosylation is unclear. As described above, both selective inhibitors differ from the traditional NSAIDs in that they are able to interact with a slightly different region of the COX-2 active site. The traditional NSAIDs also tend to have a higher affinity for COX-1 than COX-2, whereas etoricoxib is reported to have one of the lowest affinities for COX-1, even lower than celecoxib's [40]. However, within the context of our study, etoricoxib actually appears to have more in common with the traditional NSAIDs than with celecoxib. We speculate that the difference found between these two selective inhibitors may be due to the fact that although celecoxib has a lower affinity for COX-1, its affinity for COX-2 is much higher than that of etoricoxib's, as reported by Riendeau and colleagues [40] and confirmed in Figures 1 and 2. The affinity celecoxib has for COX-2 might be so great as to offset any small changes in conformation that glycosylation would bring about.

Since the overexpression of COX-2 has been implicated in a variety of maladies ranging from "simple" inflammation to rheumatoid arthritis and from colon cancer to Alzheimer's disease, the medical

community has embraced the use of COX inhibitors for the treatment or prevention of such pathophysiological conditions. Unfortunately, as with all drugs, the use of NSAIDs and the more selective COX-2 inhibitors (such as celecoxib, etoricoxib, rofecoxib, and meloxicam) has its risks. As mentioned previously, NSAIDs such as aspirin can lead to significant gastrointestinal problems. Selective inhibitors, on the other hand, have been linked to an increased risk of heart attacks and other cardiovascular side effects [41,42]. Clearly, a greater understanding of how COX inhibitors interact with COX-2 is urgently needed in order to provide better treatment options for those suffering from COX-2-related diseases and conditions. Our data suggest that the effectiveness of certain inhibitors may depend greatly upon which glycoforms are expressed. Unfortunately, there are virtually no studies describing the type of COX-2 glycoforms that are overexpressed in any of the COX-2-related diseases. Our present study indicates that such an analysis is necessary and long overdue. It is no longer enough to report the overexpression of COX-2. Also determining the expression levels of specific COX-2 glycoforms could have tremendous therapeutic implications, particularly regarding what type of COX-2 inhibitor to use and at which concentration(s) in order to effectively treat or prevent diseases such as arthritis or colon cancer.

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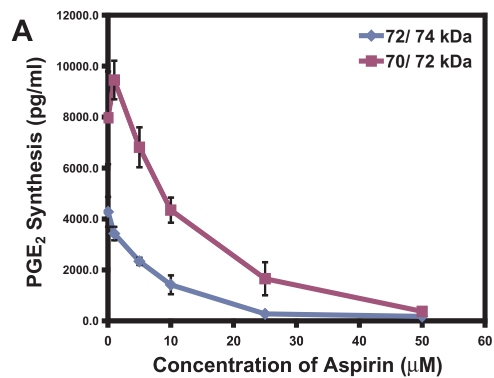
Figure Legends

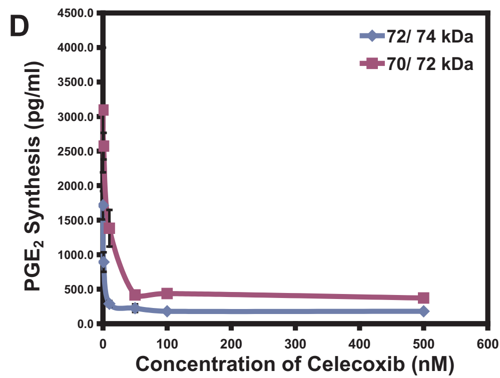
Fig. 1. Determining effective inhibitor concentration range for linear regression analyses. COS-1 cells transfected with either the wild-type (72/74 kDa) or mutant (70/72 kDa) human COX-2 gene were treated for 1 hour with: *A*, 0- 50 μM aspirin; *B*, 0- 20 μM flurbiprofen; *C*, 0- 20 μM ibuprofen; *D*, 0- 500 nM of the selective COX-2 inhibitor, celecoxib; or *E*, 0- 500 nM of the selective COX-2 inhibitor, etoricoxib. Cells were then incubated with 5 μg/ml of the COX-2 substrate, AA. The media were removed and analyzed for PGE_2 using ELISA. Results are representative of 2- 3 separate experiments. Data are presented as averages ±SEM; n = 3.

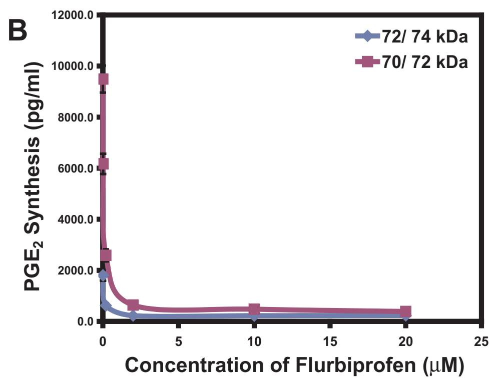
Fig. 2. Effect of COX-2 glycosylation on the efficacy of various COX-2 inhibitors. Experimental design was identical to that described in Fig. 1 except that inhibitors were analyzed over a narrow concentration range. Graphs depict linear regression analyses of PGE_2 levels from COX-2-transfected cells treated with: *A*, 0- 10 μM aspirin; *B*, 0- 0.10 μM flurbiprofen; *C*, 0- 0.80 μM ibuprofen; *D*, 0- 5 nM celecoxib; or *E*, 0- 50 nM etoricoxib. Equations for each linear regression are shown for the 72/74 and 70/72 kDa glycoforms. Results are representative of 4- 5 separate experiments. Data are presented as averages ±SEM; n = 3.

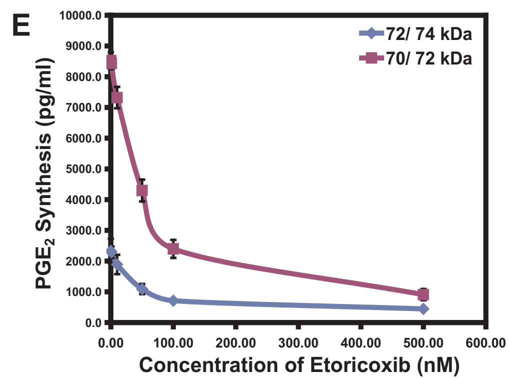
Fig. 3. Effect of inhibitor treatment on COX-2 protein expression. COS-1 cells transfected with either the wild-type or mutant COX-2 gene were treated for 1 hour with inhibitors at the narrow concentration ranges described in Fig. 2. This was followed by a 2-hour treatment with the COX-2 substrate, AA. Cells were lysed and 80 μg of total protein was loaded per lane on a 7% SDS-polyacrylamide gel and COX-2 and β-actin expressions were analyzed using Western blotting.

FIGURE 1









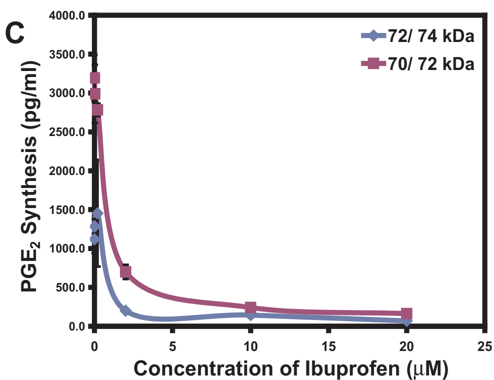
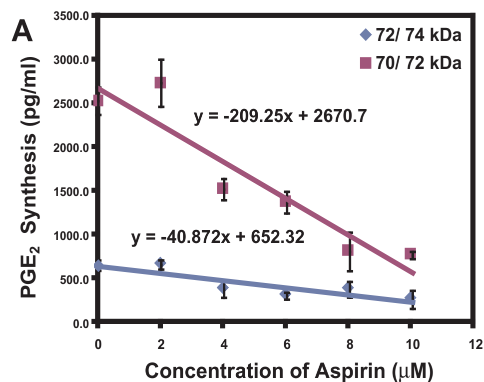
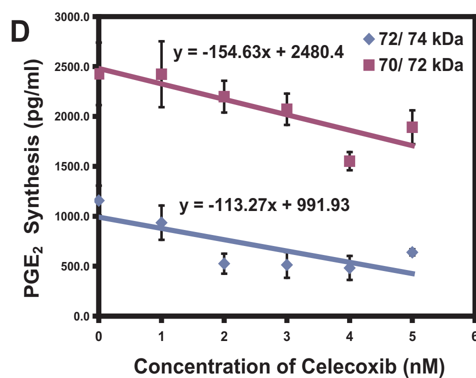
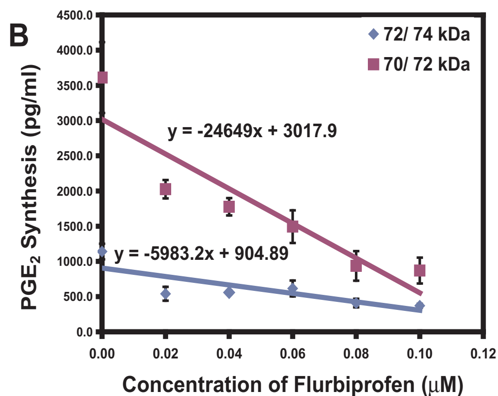
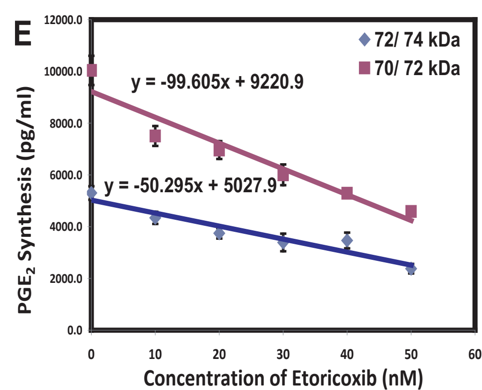


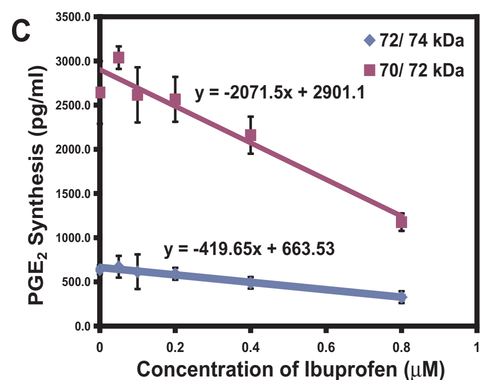
FIGURE 2

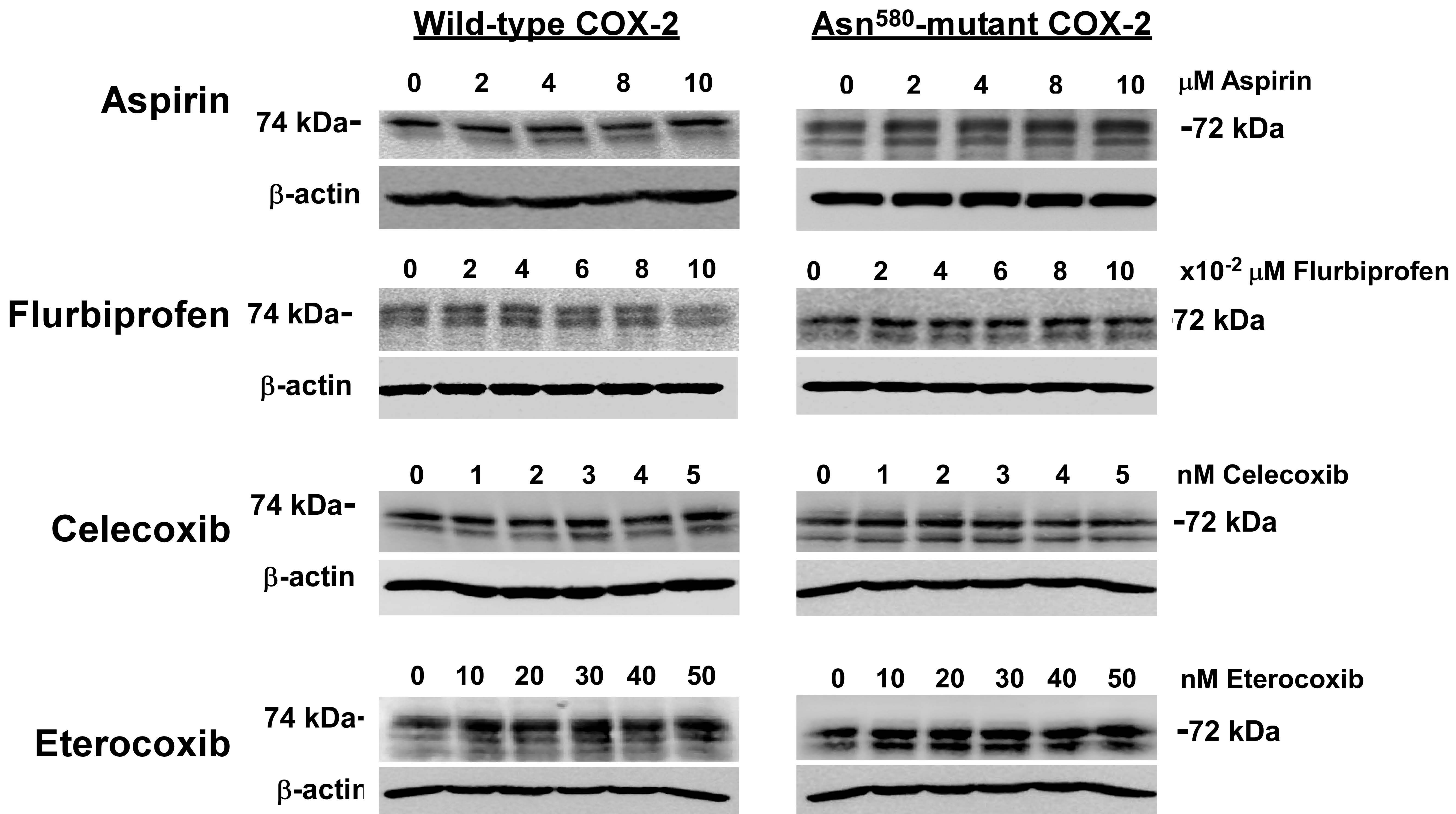











Wild-type COX-2
Asn580-mutant COX-2
Aspirin
0 2 4 8 10
μM Aspirin
74 kDa-
-72 kDa
β-actin
Flurbiprofen
0 2 4 6 8 10
x10-2 μM Flurbiprofen
74 kDa-
72 kDa
β-actin
Celecoxib
0 1 2 3 4 5
nM Celecoxib
74 kDa-
-72 kDa
β-actin
Eterocoxib
0 10 20 30 40 50
nM Eterocoxib
74 kDa-
-72 kDa
β-actin