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GLYCOSYLATION REGULATES TURNOVER OF CYCLOOXYGENASE-2

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Abstract

Cyclooxygenase-2 (COX-2) catalyzes the rate-limiting step in the prostanoid biosynthesis pathway, converting arachidonic acid into prostaglandin H_2. COX-2 exists as 72 and 74 kDa glycoforms, the latter resulting from an additional oligosaccharide chain at residue Asn^{580}. In this study, Asn^{580} was mutated to determine the biological significance of this variable glycosylation. COS-1 cells transfected with the mutant gene were unable to express the 74 kDa glycoform and were found to accumulate more COX-2 protein and have five times greater COX-2 activity than cells expressing both glycoforms. Thus, COX-2 turnover appears to depend upon glycosylation of the 72 kDa glycoform.

Keywords: cyclooxygenase-2; post-translational regulation; enzyme turnover; glycosylation; glycoforms; site-directed mutagenesis

Abbreviations: COX, cyclooxygenase; 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; OA, octanoic acid; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; NSAIDs, nonsteroidal anti-inflammatory drugs; ER, endoplasmic reticulum

1. Introduction

Prostanoids, which consist of prostaglandins and thromboxanes, represent a family of lipid-soluble, bioactive compounds which have been associated with a multitude of physiological processes and pathophysiological conditions that include: platelet aggregation, bone metabolism, ovulation, inflammation, ischemia, and various cancers (reviewed in [1]). The rate-limiting step in the prostanoid synthesis pathway is catalyzed by the integral membrane protein cyclooxygenase (COX), also known as prostaglandin H_2 synthase [2,3], which is localized to the endoplasmic reticulum (ER) and nuclear envelope [4]. COX is bifunctional, converting arachidonic acid (AA), an omega-6 fatty acid, to the precursor prostaglandin G_2 (PGG_2) and subsequently converting PGG_2 to the precursor prostaglandin H_2 (PGH_2) via cyclooxygenase and peroxidase activities, respectively. Three isoforms of COX have been found— the constitutively expressed COX-1 [5-7]; COX-2, which can be inducible or constitutive, depending on the tissue [1,8-10]; and the constitutively expressed COX-3, which is believed to be a splice variant of COX-1 [11]. Although COX-1 is considered the housekeeping enzyme expressed in nearly all tissues, COX-2 is generally perceived to be involved in pathological conditions, such as inflammation and cancer [1,12,13]. In particular, we have found that the presence of AA in human prostate cancer cells up-regulates COX-2 mRNA and protein expression [14,15]— specifically, the 72 kDa glycoform [16]. However, COX-2 is also involved with normal physiological processes such as neurotransmission and synaptic activity [17,18], maintaining normal renal functions [19], providing vascular protection [20], regulating cerebral blood flow [21], and facilitating pregnancy [22].

The COX-2 sequence contains five potential N-glycosylation sites, three of which are always glycosylated, one (Asn^{580} in human and mouse) that is glycosylated $\leq 50\%$ of the time, and one that is never glycosylated [23]. The carbohydrate moieties at each site are believed to be high-mannose chains [24,25]. The variability of glycosylation at Asn^{580} leads to the production of two distinct glycoforms of 72 and 74 kDa. Previous studies have examined this variable glycosylation at the Asn^{580} site, but none were able to determine the purpose or biological significance of the two COX-2 glycoforms [23,25].

In this study, we strove to determine the purpose of glycosylation at Asn^{580}— specifically, if and how this additional glycosylation affects COX-2 activity. We found that glycosylation at Asn^{580} does indeed affect total COX-2 activity by controlling the enzyme's turnover.

2. Materials and methods

2.1. Materials

The human COX-2 cDNA in plasmid pcDNA3 was generously provided by Dr. Timothy Hla from the University of Connecticut, USA. The COS-1 cell line was obtained from the UCSF Cell Culture Facility (San Francisco, CA, USA). QIAprep Spin Miniprep kit, HiSpeed Plasmid Maxi kit, and the primers used for site-directed mutagenesis (5'-TCATTAAAACAGTCACCATCCAGGCAAGTTCTTCCCGCTC-3' and 5'-GAGCGGGAAGAACTTGCCTGGATGGTGACTGTTTTAATGA-3') were purchased from QIAGEN (Valencia, CA, USA). The *Pfu*Ultra HF DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). The transfection reagent FuGENE 6 was purchased from Roche Applied Science (Indianapolis, IN, USA). One Shot TOP10 Competent *E. coli* cells, pre-made 4%- 12% Bis-Tris NuPAGE gels, and the NuPAGE system were obtained from Invitrogen (Carlsbad, CA, USA). The anti-human COX-2 polyclonal antibody, the Prostaglandin E_2 EIA kit—Monoclonal and peroxide-free arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI, USA). Octanoic acid was purchased from Sigma.

2.2. Site-directed mutagenesis

DNA primers were designed to convert the Asn residue at site 580 in the human COX-2 sequence to a Gln residue. The mutagenesis reaction contained pcDNA3-COX-2, sense and antisense DNA primers, dNTP mix, and *Pfu*Ultra HF DNA polymerase. The reaction was incubated at 95°C for 30 seconds. This was followed by 16 cycles of: 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 7 minutes, 30 seconds. The restriction enzyme *Dpn*I was then added to the reaction to cleave up the original plasmid, leaving intact only plasmids containing the mutant COX-2 gene.

2.3. 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, 1% FBS media at 37°C. FuGENE 6 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 4- 5 hours in the presence of the FuGENE 6/COX-2 DNA complex. Media was replaced with DMEM, 10% FBS, 4 mM L-glutamine, and antibiotics, and the cells continued their incubation.

2.4. Western blot analyses

Transfected and nontransfected COS-1 cells growing on 6-well plates were washed with ice-cold PBS and then lysed. The whole cell lysates were sonicated briefly and subjected to centrifugation at 14,000x g for 5 minutes to remove cytoskeletal structures. Protein concentrations were determined, and cell lysate samples underwent gel electrophoresis using either 4%- 12% Bis-Tris NuPAGE gels or 16 cm, 7% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose and immunostained for COX-2 protein using anti-human COX-2 polyclonal antibody. The membrane was also immunostained for the housekeeping gene β-actin. Bound antibody was detected using chemiluminescence and film. Densitometry analyses were then carried out using the software UNSCAN-IT by Silk Scientific.

2.5. ELISA for measuring PGE_2 levels

Transfected and nontransfected COS-1 cells growing on 6-well plates were treated with 5 μg/ml of either AA or OA for 2 hours at 37 °C, as previously described [16]. Media samples were then collected and analyzed for the presence of the downstream product prostaglandin E_2 using a

Prostaglandin E_2 EIA kit—Monoclonal. Dilutions of the media samples were prepared in a 96-well plate and were treated and analyzed according to the manufacturer's instructions.

2.6. Statistical analysis

Using Instat 3 software, densitometry data were subjected to One-way Analysis of Variance (ANOVA). ELISA results were also subjected to ANOVA followed by the Student-Newman-Keuls Multiple Comparisons Test.

3. Results and discussion

3.1. Mutagenesis of glycosylation site Asn^{580} affects glycoform expression

By replacing asparagine (Asn) with glutamine (Gln) at residue 580 of the human COX-2 gene, we effectively eliminated glycosylation at that site (Fig. 1*A*). As a result, COS-1 cells transfected with the mutant gene were able to express the 72 but not the 74 kDa glycoform found in cells expressing the wild-type gene. The mutation also resulted in the expression of a "new" 70 kDa glycoform, but the mechanism behind its formation is as yet unknown. Fig. 1*A* shows a significantly large accumulation of both the 70 and the 72 kDa proteins. In short, removal of the glycosylation site appeared to increase total COX-2 protein levels. To confirm this, a timecourse experiment was conducted in which expression patterns of the glycoforms were analyzed 3, 4, and 5 days after transient transfection with either the wild-type or mutant COX-2 gene (Fig. 1*B*). The concentrations of the two glycoforms expressed from the mutant gene continued to be greater than those expressed from the wild-type gene, even as total COX-2 expression started to wane by Day 5. This verified that removal of the Asn^{580} glycosylation site slowed down the turnover of the COX-2 protein.

3.2. Effect of glycosylation on total COX-2 activity

To determine if the accumulation of COX-2 in cells expressing the mutant gene also resulted in an increase in total COX-2 activity, levels of the downstream end-product prostaglandin E_2 (PGE_2) were

measured (Fig. 2). Cells carrying either the wild-type (WT) or mutant (MUT) COX-2 gene were treated with the COX-2 substrate AA for 2 hours. PGE_2, released by cells into the media, was measured using an ELISA. As Fig. 2 shows, AA-treated cells expressing the 70/ 72 kDa glycoforms had the greatest PGE_2 levels— five times greater than the levels found in AA-treated cells expressing the 72/ 74 kDa glycoforms. PGE_2 was also measured in untreated cells and in cells treated with the non-substrate fatty acid, octanoic acid (OA). As expected, PGE_2 levels were extremely low in the untreated and OA-treated cell groups, and there were no significant differences between the two groups.

3.3. Effect of AA on COX-2 glycoform expression

Although the presence of AA can lead to an increase in COX-2 by indirectly up-regulating its transcription [15,26], the mutant and wild-type COX-2 gene constructs used in this study were under the control of the pcDNA3 plasmid's CMV promoter rather than their native COX-2 promoter. Thus, the increased COX-2 activity in AA-treated cells (shown in Fig. 2) was not due to an increase in COX-2 production. However, to confirm that AA treatment had no effect on COX-2 protein synthesis, a Western blot was carried out on the same treatment groups described in Fig. 2. Fig. 3 shows that AA-treated cells produced the same level of COX-2 protein as control cells (i.e. untreated and OA-treated cells).

3.4. Conclusion

A few past studies have examined the glycosylation of COX-2, but none succeeded in determining the significance or purpose of the two COX-2 glycoforms. Though researchers found glycosylation of COX-2 at Asn^{53}, Asn^{130}, and Asn^{396} necessary for proper folding of COX-2 into an active, 72 kDa enzyme [23], no such function was found for the glycosylation site Asn^{580}. Ours is the first study to describe a biologically significant role for the glycosylation of Asn^{580} in the COX-2 protein. Additionally, our study introduces a new regulatory mechanism for COX-2 expression. Regulation of COX-2 at the levels of transcription (reviewed in [27]) and post-transcription (i.e. mRNA stability) [28,29] has already been well-established. In fact, we previously demonstrated that AA regulates COX-2

transcription via a feed-forward mechanism in prostate cancer [15,26], most likely mediated by the EP4 prostaglandin receptor [30] and by activation of phosphatidylinositol 3-kinase (PI3K) [16]. However, regulation after protein synthesis appeared to be limited to the addition of exogenous COX-2 enzyme inhibitors (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs)) [1]. As our study indicates, removal of glycosylation site Asn^{580} in the human COX-2 protein leads to an increase in total COX-2 activity (as reflected by a five-fold increase in PGE_2) and an accumulation of both the 72 kDa and 70 kDa COX-2 glycoforms. Although the structure and activity level of the 70 kDa protein have yet to be determined, it is entirely possible that this glycoform contributed significantly to the overall increase in COX-2 activity found in the Asn^{580}-mutant cells. All these data suggest that normal turnover of COX-2 requires the conversion of the 72 kDa protein into the 74 kDa glycoform via glycosylation, indicating that regulation of COX-2 can also occur at the post-translational level. This finding may be particularly important from a therapeutic perspective since increased levels of COX-2 protein have been implicated in various pathological conditions, and glycosylation of COX-2 appears to be involved with controlling those levels.

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Figure legends

Fig. 1. COX-2 glycoforms expressed from wild-type and mutant COX-2 genes. *A,* Whole cell lysates were analyzed via Western blotting after transient transfection of COS-1 cells with either the wild-type or Asn^{580}-mutant COX-2 gene. Each lane was loaded with 15 µg of protein. Blot shown represents a sample size of ten (n = 10). Graph depicts densitometry values of COX-2 bands normalized to β-actin levels. *** $p<0.0001$ compared to WT 72 kDa; n = 3. *B,* Whole cell lysates were analyzed 3, 4, and 5 days after transient transfection with the wild-type or mutant COX-2 gene. Each lane was loaded with 15 µg of protein. Blot shown is representative of duplicate samples (n = 2). Graphs depict densitometry values of COX-2 bands normalized to β-actin levels. ANOVA p values: p=0.0116 for WT 74 kDa; p=0.0030 for WT 72 kDa; p=0.0049 for MUT 72 kDa; and p=0.0005 for MUT 70 kDa; n = 3 for 3-day data; n = 2 for 4- and 5-day data. **C**, nontransfected control cells; **WT**, cells transfected with the wild-type COX-2 gene; **MUT**, cells transfected with the mutant gene.

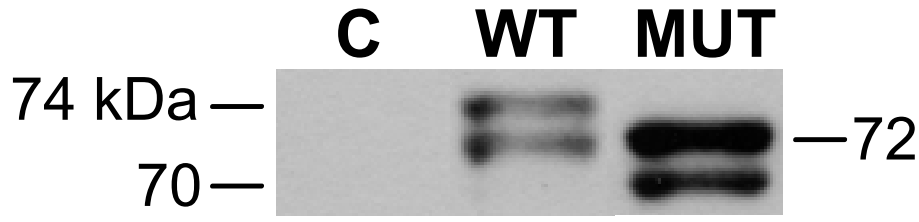
Fig. 2. Comparing activities of COX-2 glycoforms in the presence of arachidonic or octanoic acid. Three days after transient transfection of COS-1 cells with either the wild-type (**72/ 74 kDa**) or mutant (**70/ 72 kDa**) COX-2 gene, cells were treated with 5 µg/ml of arachidonic acid (**AA**) or octanoic acid (**OA**) for 2 hours at 37°C. Media was then analyzed for PGE_2 using an ELISA. **Neg. Control**, nontransfected cells. Data are represented as average ± SEM. ***** $p < 0.05$ compared to other two "AA-treated" groups; ****** $p < 0.001$ compared to other two "72/ 74 kDa" groups; n = 3.

Fig. 3. Effect of arachidonic and octanoic acid on COX-2 protein expression. Three days after transient transfection of COS-1 cells with either the wild-type (**WT**) or mutant (**MUT**) COX-2 gene, cells were treated with 5 µg/ml of arachidonic acid (**AA**) or octanoic acid (**OA**) for 2 hours at 37°C. Whole cell lysates were then analyzed via Western blotting, each lane loaded with 10 µg of protein. Blot shown is representative of triplicate samples (n = 3). Graph depicts densitometry values of COX-2 bands

normalized to β-actin levels. No statistically significant differences were found between nontreated (**1**), AA-treated (**2**), or OA-treated (**3**) samples for either the WT or MUT groups; n = 3.

FIGURE 1

A


C
WT
MUT
74 kDa
70
72

B

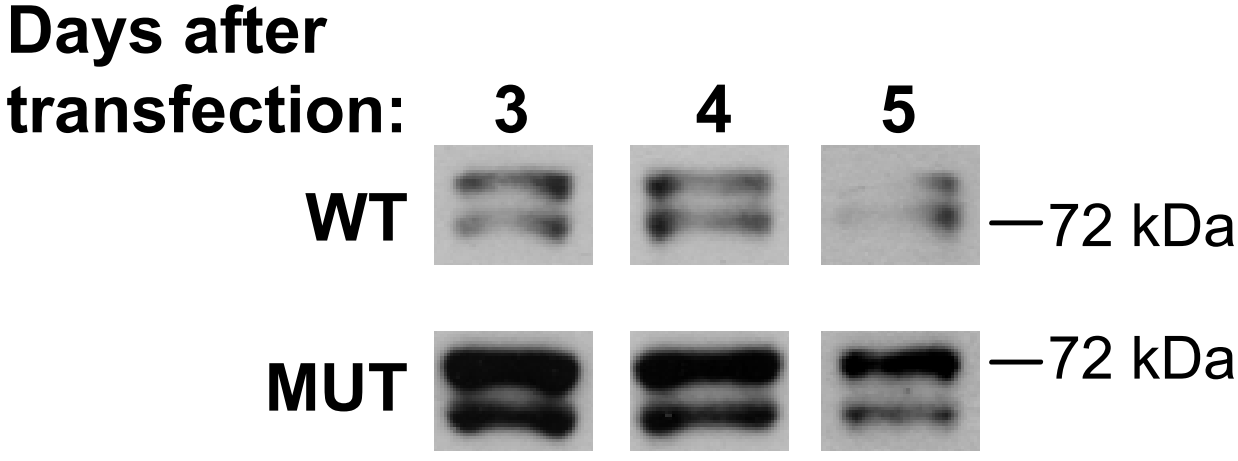

Days after transfection:
3
4
5
WT
72 kDa
MUT
72 kDa

FIGURE 2

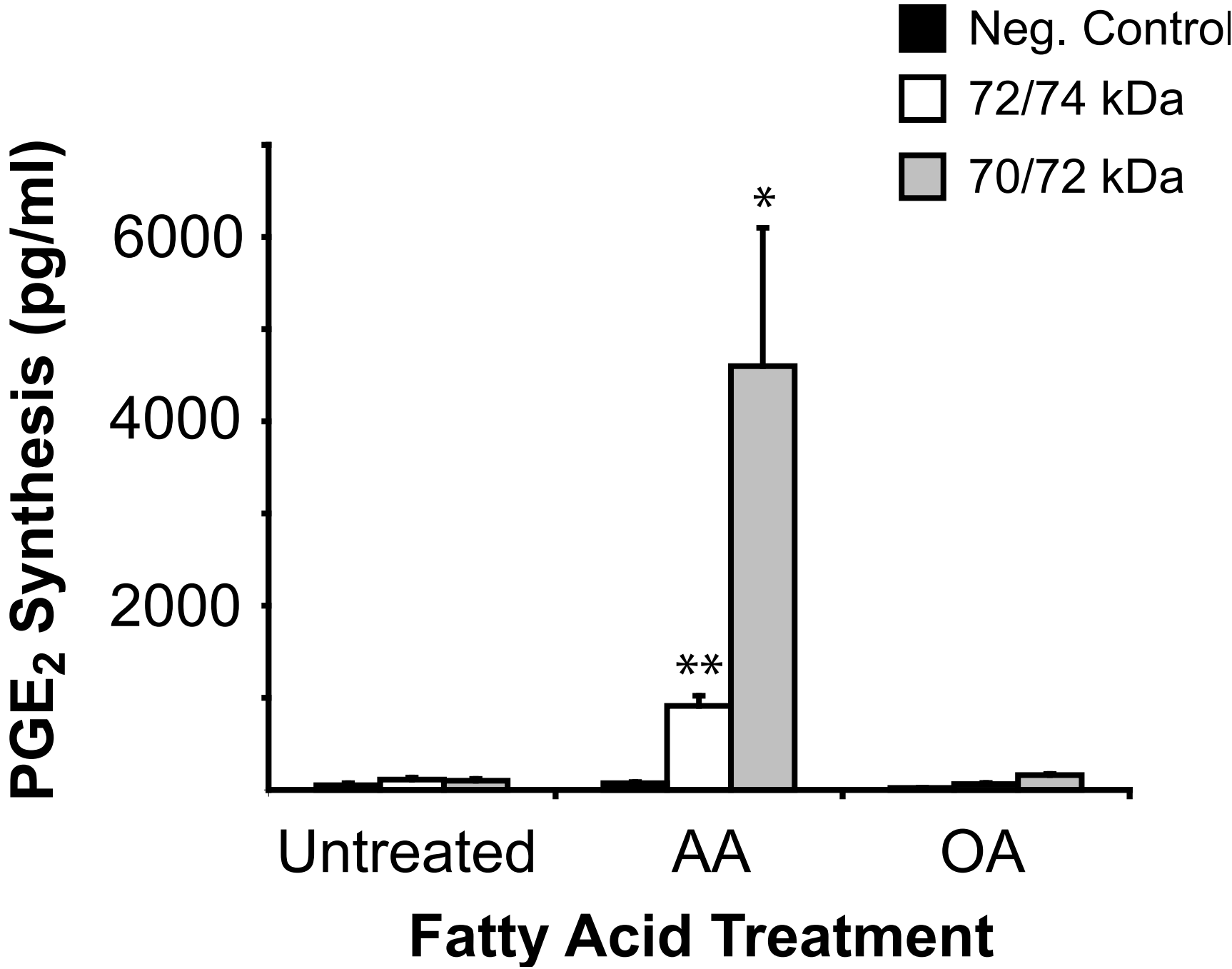
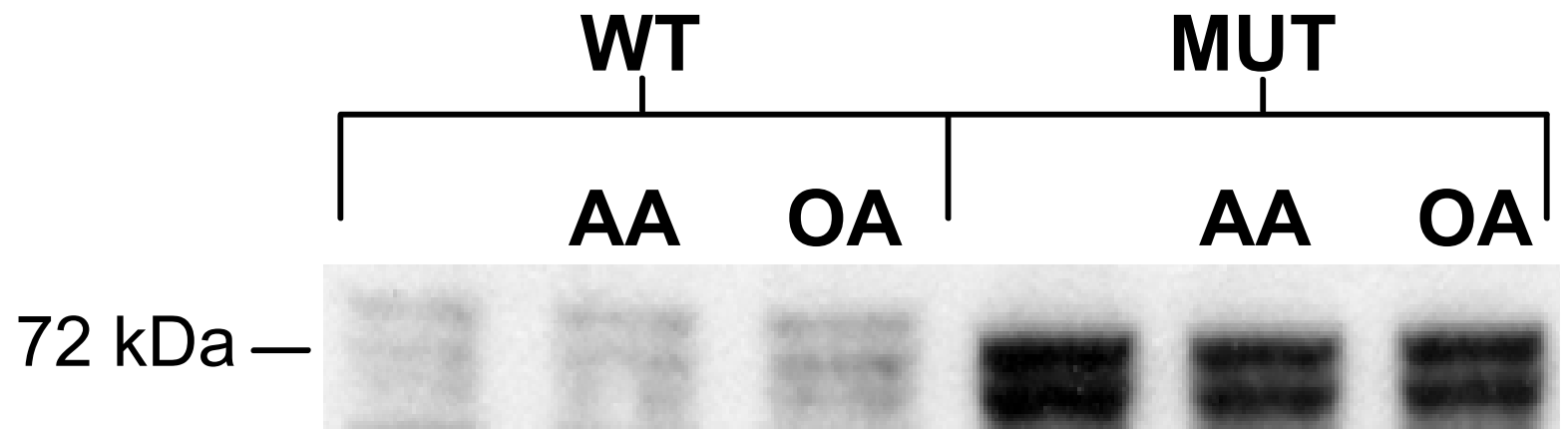
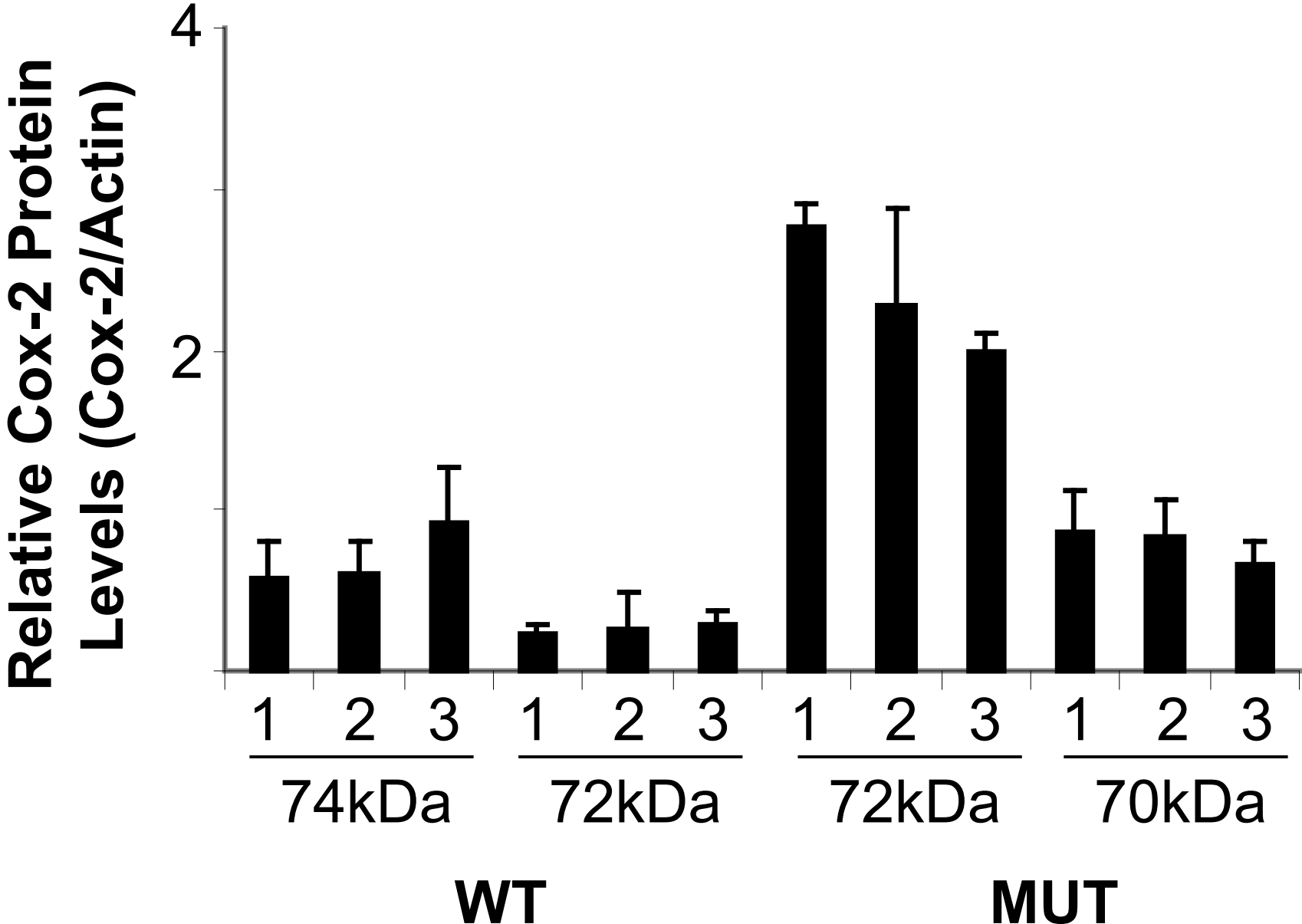

Neg. Control
72/74 kDa
70/72 kDa
PGE_2 Synthesis (pg/ml)
6000
4000
2000
*
**
Untreated
AA
OA
Fatty Acid Treatment

FIGURE 3


WT
MUT
AA
OA
AA
OA
72 kDa


Relative Cox-2 Protein Levels (Cox-2/Actin)
4
2
1 2 3 1 2 3 1 2 3 1 2 3
74kDa
72kDa
72kDa
70kDa
WT
MUT