Low Prostaglandin E_2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics

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Summary at a glance

We demonstrate that the role of cyclooxygenase enzymes in arachidonic acid metabolism is altered in the nasal mucosa of aspirin-intolerant asthmatic patients. This suggests that abnormalities in cyclooxygenase enzymes are present in all the nasal mucosa of these patients. Our results provide further support to the consensus term of rhinosinusitis.

ABSTRACT

Background and objective: Anomalies in the regulation of cyclooxygenase (COX)-1 and-2 have been described in nasal polyps of aspirin-induced asthma (AIA). Whether these anomalies are specific to nasal polyps or affect all the nasal mucosa (NM) of upper airways is still unclear. The objective of this study was to compare the COX pathway in NM of AIA patients with the NM of control subjects.

Methods: Fibroblasts were isolated from NM of 5 AIA patients (AIA-NM) and 5 control subjects (control-NM). Cells were treated with 10 ng/ml IL-1β for up to 72 hours. Prostaglandin E_2 (PGE_2) production was measured by ELISA, expression of COX-1 protein by Western blot, and COX-2 protein by ELISA, Western blot and immunofluorescence techniques.

Results: IL-1β increased PGE_2 production and COX-1 protein expression in control-NM fibroblasts, but no changes were found in AIA-NM. IL-1β provoked a significant time-dependent increase in COX-2 protein expression in control-NM fibroblasts but had a very mild effect on COX-2 protein expression in AIA-NM.

*Conclusions***:** Our data suggest that abnormalities in the COX pathway are not a phenomenon exclusive to nasal polyp mucosa as they are also present in all the nasal mucosa of AIA patients. These anomalies may be involved in the pathogenesis of airway inflammation and NSAID intolerance in asthma patients with chronic rhinosinusitis and nasal polyposis.

Key words: Aspirin intolerance, cyclooxygenase, fibroblasts, nasal mucosa, prostaglandin E_2

Short Title: COX Pathway in ASA-sensitive Asthma

INTRODUCTION

Aspirin-induced asthma (AIA) is a syndrome characterized by the association of asthma, chronic rhinosinusitis with nasal polyps (NP), and episodes of bronchospasm precipitated by non-steroidal anti-inflammatory drugs (NSAIDs).[1] Although the mechanism responsible for AIA has not been completely elucidated, anomalies in the regulation of arachidonic acid (AA) metabolism seem to be involved in the adverse response to NSAIDs in these patients.[1,2]

Upon stimulation, activated phospholipase A_2 releases AA from membrane phospholipids. AA is then converted by enzymatic and non-enzymatic pathways into a large number of eicosanoids. The major enzymatic routes include the lipoxygenase (LO) pathway, which is responsible for the formation of leukotrienes, 15-HETE, and lipoxins; the cyclooxygenase (COX) pathway, involved in the formation of prostaglandins (PG), thromboxanes and prostacyclin; and the cytochrome P450 pathway, responsible for the formation of 20-HETE and other metabolites.[3,4]

In the human lung, the 5-LO pathway is responsible for the synthesis of cysteinyl-leukotrienes (CysLTs). Various studies have reported that this pathway is up-regulated in the upper and lower airways of AIA patients. The expression of the enzyme LTC_4 synthase, which is involved in the synthesis of LTC_4, is much higher in the airways of AIA patients than in those of aspirin-tolerant asthma patients and healthy controls.[5,6] The increased activity of the 5-LO pathway results in overproduction of CysLTs in AIA, which is further increased when patients are exposed to NSAIDs.[7,8]

This over-expression of CysLT is a finding reported by almost all the authors who have studied the mechanism of AIA. However, the presence of anomalies in the regulation of the COX metabolism has been reported in only some of these studies, and their role in

the pathogenesis of AIA remains to be clarified.[1,2] Accordingly, this study has focused solely on the COX pathway in AIA.

The COX pathway converts AA into PGs, including PGE_2, PGD_2 and $PGF_{2\alpha}$. Two functional COX isoforms have been identified. COX-1 is constitutively expressed in most tissues and generates PGs which are primarily involved in the regulation of homeostatic functions. COX-2 is inducible by several cytokines and growth factors, including interleukin (IL)-1β and tumor necrosis factor-α .[3,4]

Various anomalies have been reported in the COX pathway of AIA.[1,2] Low production of PGE_2 associated with down-regulated expression of COX enzymes has been reported in NP tissue and in both fibroblasts and epithelial cells from the NP of AIA patients,[9-15] suggesting that abnormalities in AA metabolism could play a role in AIA etiology. However, there are no reports to date of such alterations in the NM of AIA patients. In contrast to the results obtained in NP, studies using bronchial biopsies and cultured bronchial fibroblasts from AIA and aspirin-tolerant asthma have not been able to find any differences in the expression of COX-1 and COX-2 when compared to healthy controls.[5,16] There are no clear explanations for the reported discrepancies between the results obtained in the upper and lower airways. These discrepancies might be due, at least in part, to the fact that the regulation of COX enzymes has been extensively studied by comparing NP with NM from control subjects[10-14] but has yet to be studied by comparing NM from AIA patients with NM from control subjects. In other words, since inflammatory polyps are structures found in the upper airways but never seen in the lower airways, it is reasonable to ask whether the anomalies reported in the regulation of COX-1 and COX-2 expression in the upper airways are specific to NP and therefore not representative of either the NM surrounding the NP structure or the bronchial mucosa.

The objective of this study was to compare the COX pathway in the NM of AIA patients with the NM of control subjects. With this aim in mind, we studied and compared PGE_2 production and COX-1 and 2 expression in fibroblasts derived from the NM of both AIA and control subjects.

METHODS

Study population

We obtained NM specimens from 5 non-asthmatic subjects with either septal deviation or turbinate hypertrophy who had undergone nasal corrective surgery (control-NM). All the control subjects had taken aspirin or NSAIDs at clinical dosage without any untoward reactions (asthma and/or rhinitis, urticaria, angioedema or anaphylaxis). NM specimens were also collected from 5 asthmatic patients with chronic rhinosinusitis and aspirin intolerance (AIA-NM) who had undergone endoscopic sinus surgery. The clinical and demographic characteristics of the subjects are shown in Table 1. The diagnosis of aspirin intolerance was confirmed by lysine-aspirin nasal challenge, as previously described.[17] None of the control subjects had had any oral or intranasal corticosteroid treatment for at least one month before surgery. None of the patients had any upper airway infection in the 2 weeks before surgery. All patients gave informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our Institution.

Tissue handling and cell culture

NM tissue was cut into 3 x 3 mm fragments and placed in six-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, California, USA) and 2 µg/ml amphotericin B (Sigma, St Louis, MO, USA). Cultures were placed in a 5% CO_2 humidified incubator at 37ºC. Once the fibroblasts had grown, tissue fragments were removed and the first passage was performed by adding 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, California, USA) for 5 min. The reaction was stopped with 10% FBS-supplemented DMEM. Cells were then centrifuged (400g, 5 min) and seeded in 150 cm^2 flasks (NUNC). At passages 3 to 7, fibroblasts were cultured in CultureSlides® and flasks to perform culture characterization and experimental protocols. The same batch of FBS was used for the whole experimental period. Mycoplasma contamination was tested by PCR in the cultures and all of them were negative.

Culture characterization

Culture characterization was performed by immunofluorescence for vimentin (fibroblasts), cytokeratins (epithelial cells) and alpha smooth muscle actin (myofibroblasts) in CultureSlides® incubated with serum-free media (SFM) for 24 hours.

Experimental protocols

When cultures reached 80% confluence, FBS-supplemented media was switched to serum-free media (SFM) for 24 hours. Cells were then incubated with SFM in the presence or absence of 10 ng/ml IL-1β (R&D Systems Minneapolis, MN, USA) for

different times. CultureSlides® were used to study COX-2 protein expression by immunofluorescence. Flask culture supernatants were centrifuged (400g, 10 min at 4ºC), sterilized through 0.22 µm filters, and stored at -80ºC until the PGE_2 concentrations were measured. Total proteins were obtained by scraping the flasks after two washes with cold PBS. The cells were centrifuged (1,500g, 5 min at 4ºC) and resuspended in 0.4 ml cold lysis buffer (Complete™ protease inhibitor tablet in 50 ml of 0.05 M Hepes buffer solution, 0.05% v/v Triton X-100, and 625 µM PMSF). Cells were sonicated twice for 15 seconds in a sonifier (Branson, Danbury, CT, USA) and centrifuged (12,000g, 10 min at 4ºC). Cell lysates were used to analyze protein expression by Western blot and ELISA.

Immunofluorescence

This technique was performed as previously reported [14]. The primary antibodies were against alpha-smooth muscle actin (M0851, DAKO, Glostrup, Denmark) at dilution 1:500, vimentin at 1:100 (V5255, Sigma, Saint Louis, Missouri, USA), pan-cytokeratin at 1:200 (C2562, recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19, Sigma) or COX-2 (SC-1745, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). The percentage of positive cells was quantified using fluorescence microscopy.

PGE_2 and COX-2 ELISA

Concentrations of PGE_2 in supernatants were measured using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA). The assay range was 7.81-1000 pg/ml. COX-2 protein concentrations were measured using an enzyme-linked immunosorbent sandwich assay (Zymed Laboratories, San Francisco, CA, USA). The assay range was

2.15-275 ng/ml. The concentrations of PGE_2 and COX-2 were normalized to the total protein content in the cell lysate from corresponding samples.

Western Blot

To analyze COX-1 and COX-2 protein expression, we performed the technique we have previously reported.[14] The primary antibodies used were against COX-1 (SC-1752, Santa Cruz) and COX-2 (SC-1745, Santa Cruz) at dilution 1:1000. Immunoreactive bands were visualized using a chemoluminiscent method (Supersignal West Pico Chemiluminescent Substrate, Rockford, IL, USA). Light emissions were detected by the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software and normalized by β-actin band intensities assessed in the same samples.

Statistical analysis

Data are reported as follows: immunofluorescence data are expressed as median and 25-75th percentile of positive cell percentage among total cells. ELISA results are shown as median and 25-75th interquartile of pg of PG or ng of COX-2/μg total proteins. Finally, Western blot results are depicted as median and 25-75th percentile of band intensities normalized by β-actin. The nonparametric statistical Mann-Whitney U-test was used for between-group comparisons and the Wilcoxon test was used for paired comparisons, after confirming differences with the Friedman test. Statistical significance was set at $P<0.05$.

RESULTS

Culture characterization

All the cells in culture had fibroblast phenotype since 100% of cells were positive to vimentin. There was a total absence of epithelial cells in the fibroblast cultures since no cytokeratin-positive cells were found. Smears of nasal mucosa epithelial cells were used as a positive control (Figure 1). No significant differences were observed in the myofibroblast percentage in fibroblast cultures (control-NM=1.6%, 1.3-3.3; AIA-NM=3.5%, 2.0-3.6, N=5).

Prostaglandin E_2 production

There were no significant differences in the production of PGE_2 at baseline in cultured fibroblasts derived from either control-NM or AIA-NM. Compared to SFM-treated cells, IL-1β significantly increased PGE_2 production at 24 hours in control-NM, but had no effect on PGE_2 secretion in fibroblasts derived from AIA-NM (Figure 2).

COX expression

COX-1 basal expression was not different between control-NM and AIA-NM. Compared to SFM-treated cultures, however, IL-1β induced a significant time-dependent increase in COX-1 protein expression in control-NM fibroblasts from 24 to 72 hours. In contrast, a tendency to decreased COX-1 expression was found in AIA-NM fibroblasts (Figure 3).

COX-2 expression was measured by ELISA, Western blot and immunofluorescence techniques. COX-2 protein expression was not detected in SFM-treated cultures, because the level of the protein was under the level of detection of the ELISA. No cells

showed fluorescence and no detectable signals were present in Western blot. When cells were incubated with IL-1β, however, there was a significant time-dependent increase in COX-2 expression from 2 to 24 hours (measured by ELISA) in control-NM fibroblasts. In contrast, IL-1β had a very mild and non-significant effect on the induction of COX-2 in AIA-NM fibroblasts (Figure 4A). Similarly, when analyzed by Western blot and compared to SFM treated cultures, IL-1β increased the ratio of COX-2/β-actin protein expression only in control-NM (Figure 4B). Finally, when studied by immunofluorescence, there was a significant increase in COX-2 positive cells in response to IL-1β in both control-NM and AIA-NM cultures. The percentage of COX-2 positive cells in IL-1β-treated cultures was significantly higher, however, in control-NM than in AIA-NM fibroblasts (Figure 4C, D).

DISCUSSION

The main findings of our study were: (1) IL-1β markedly stimulated the production of PGE_2 in control-NM fibroblasts but had a non-significant effect on fibroblasts obtained from AIA-NM; (2) IL-1β stimulation increased COX-1 protein expression in fibroblasts isolated from control-NM, but not in fibroblasts obtained from AIA-NM; and (3) there was a marked increase in COX-2 protein expression following IL-1β exposure in control-NM fibroblasts, but almost no effect on fibroblasts derived from AIA-NM.

This is the first study to demonstrate anomalies in the COX pathway of AA metabolism in nasal mucosa from AIA patients. Previous studies have reported decreased production of PGE_2 in nasal polyps – an anomaly that is even more marked in the nasal

polyps of patients with AIA.[10,11,16] In line with this finding, various studies have reported a lack of up-regulation of COX-2 in nasal polyps from both aspirin-tolerant asthma and AIA.[11-15,18] For reasons that remain unclear, other studies have been unable to find any differences in the expression of COX-2 between nasal polyps and control nasal mucosa.[6,19] As most of the studies that did not find any differences in the expression of COX-2 in AIA were performed with immunohistochemistry techniques, we used three different methods of analysis (ELISA, Western blot and immunofluorescence) to assess the level of COX-2 expression.

In our study, the three methods presented similar outcomes and confirmed that COX-2 protein is not detected in non-stimulated fibroblasts and almost fails to be up-regulated by inflammatory stimuli in the nasal mucosa of AIA. These results are in keeping with other studies showing undetectable COX-2 protein in unstimulated nasal mucosa and polyp fibroblasts.[14] In contrast, increased expression of COX-2 has been reported in nasal polyps from cystic fibrosis patients, as expected in inflammatory diseases.[20,21] This observation adds further support to the notion that, for reasons that are still unknown, COX-2 is abnormally regulated in the nasal mucosa of patients with AIA.

Previous studies suggest that an alteration in the regulation of COX-1 may also coexist with anomalies in the regulation of COX-2 in the nasal polyps of patients with and without associated AIA.[14,18] In this study we report that these anomalies are also present in fibroblasts derived from the nasal mucosa of AIA patients. Although it is generally accepted that COX-1 plays a limited role in inflammatory responses, mild modifications can occur in the expression of the enzyme in cells and tissues when they are stimulated by pro-inflammatory cytokines or injury.[22] The induction of COX-1 in inflammatory conditions might help to mitigate, at least in part, the deficient up-regulation of COX-2

in the mucosa of AIA patients. However, this compensatory mechanism could not occur in NM fibroblasts obtained from AIA patients as COX-1 is not induced by IL-1β.

PGE_2 exerts various anti-inflammatory and anti-fibrotic effects, including suppression of eosinophil infiltration, reduction of CysLT release, inhibition of fibroblast proliferation, myofibroblast transformation and collagen synthesis.[23]. The limited production of PGE_2 found in the NM of AIA patients may contribute to the intensification of the inflammatory process in their airways and may account for the reported association of aspirin intolerance with increased asthma and nasal-polyp severity.[10,24,25]

Moreover, when the COX-2 and PGE_2 concentrations obtained by ELISA in cultures treated with IL-1β were compared, the values obtained in the NM of AIA were similar to those previously reported in NP of AIA patients[14], indicating that these alterations are present to a similar degree in all the nasal mucosa of AIA patients.

In contrast, our study reported down-regulation of arachidonic acid metabolism in fibroblasts of nasal mucosa from AIA patients. In keeping with our findings, several studies have already reported that the COX pathways are also abnormally regulated in nasal[9] and bronchial[26] epithelial cells from aspirin-sensitive asthmatics.

The mechanisms responsible for the reported alterations in the regulation of COX-2 found in our study remain to be clarified. It is well known that inflammatory stimuli induce cellular responses through the activation of mitogen-activated protein kinases (MAPKs), and that COX-2 gene expression is also regulated by the action of

transcription factors such as NF-κB and C/EBP, among others. The role of these proteins has recently been studied in AIA-NP fibroblasts and showed no differences in MAPK phosphorylation and transcription factor nuclear translocation, compared to control nasal mucosa cells.[27] These findings suggest that the mechanisms involved in COX down-regulation should be related to other mechanisms, such as transcription factor bindability to gene promoter, the activity of histone acetyltransferases and deacetylases, and mRNA stability.

Furthermore, the absence of any differences in the myofibroblast percentage in cultures suggests that changes in cell phenotype do not play a role in the COX pathway abnormalities found in our study.

Finally, our findings, along with data from previous studies showing that IL-5 and ECP levels are increased in both sinusal (ethmoidal) and nasal (lower turbinate) samples from patients with chronic rhinosinusitis and NP,[28] give further support to the consensus term of rhinosinusitis recommended by the European Position Paper on Rhinosinusitis and Nasal Polyps (EP^3OS).[29]

In conclusion, we found significant differences in the regulation of PGE_2, COX-1 and COX-2 in the nasal mucosa fibroblasts of patients with AIA. Our data suggest that abnormalities in the COX pathway are present in all the nasal mucosa of AIA patients and are not exclusively confined to the nasal polyp mucosa. This study also demonstrates that the nasal mucosa of AIA patients cannot be used as surrogate of healthy nasal mucosa.

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Table 1. Epidemiological characteristics of control subjects and AIA patients.

Characteristics	Control-NM	NM-AIA
Fibroblast cultures, N	5	5
Age, years (mean±sem)	32.2±4.2	45.2±4.0
Female, N (%)	1 (20)	3 (60)
Asthma, N (%)	0 (0)	5 (100)
Aspirin intolerance, N (%)	0 (0)	5 (100)
STP positive, N (%)	1 (20)	0 (0)
Blood eosinophilia, % (mean±sem)	1.7±0.5	9.8±1.7
CRS with nasal polyps, N (%)	0 (0)	5 (100)
Intranasal corticosteroid, N (%)	0 (0)	3 (60)

AIA, aspirin-intolerant asthmatic.
CRS, chronic rhinosinusitis.
NM, nasal mucosa.
SPT, skin prick test.

Figure Legends

Figure 1. Culture characterization.

Representative immunofluorescent staining in control nasal mucosa fibroblasts. **A)** Fibroblast phenotype of cells in culture was confirmed by the presence of vimentin fibers. **B)** Myofibroblast phenotype of cells in culture was confirmed by the detection of α-smooth muscle actin fibers. **C)** Absence of epithelial cells demonstrated by the absence of cytokeratin staining in cultures. Original magnification x 200.

Figure 2. Time course of IL-1β effects on PGE_2 protein production

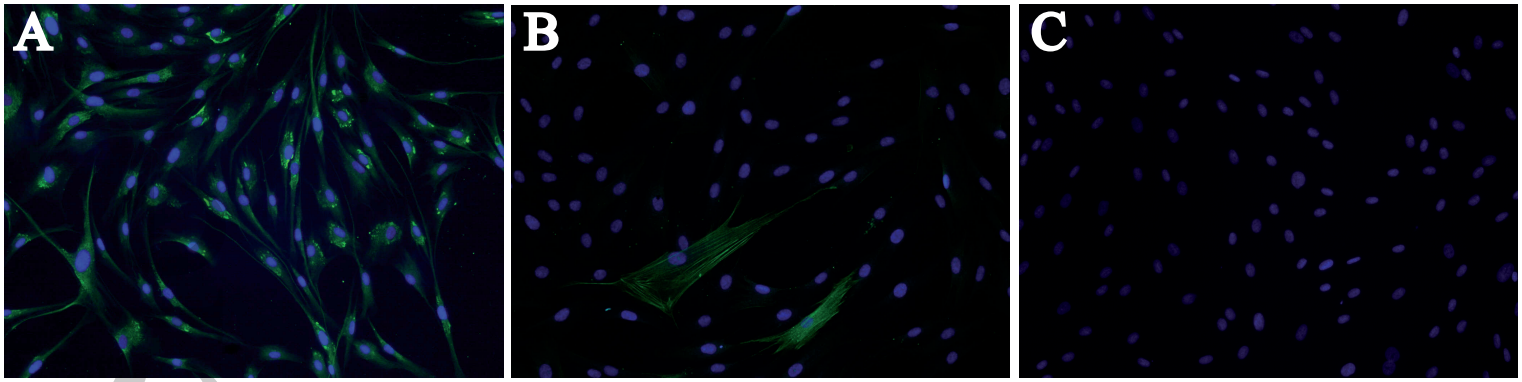
Fibroblasts were incubated in serum-free media with IL-1β at 10 ng/ml for 4 and 24 hours. PGE_2 (N=5) concentration was measured by ELISA. Wilcoxon signed-rank test was used for analysis. * $P<0.05$ compared to non-stimulated cells. AIA, aspirin-induced asthma; NM, nasal mucosa.

Figure 3. Effects of IL-1β on COX-1 protein expression.

Fibroblasts were incubated in serum-free media with IL-1β at 10 ng/ml for up to 72 hours (N=5). COX-1 protein expression was analyzed by Western blot. Mann-Whitney U-test was used for between-group comparisons and Wilcoxon test for paired comparisons. *, $P<0.05$ compared to non-stimulated cells (ratio COX-1/β-actin=1); †, $P<0.05$ and ††, $P<0.01$ compared to IL-1β–treated control-NM. AIA, aspirin-induced asthma; NM, nasal mucosa.

Figure 4. Effects of IL-1β on COX-2 protein expression.

Fibroblasts were incubated in serum-free media with IL-1β at 10 ng/ml (N=5). **A)** Time course of IL-1β on COX-2 protein expression analyzed by ELISA. Immunofluorescence **(B)** and Western blot **(C)** analysis of COX-2 protein expression in cultures incubated for 24 hours with IL-1β. **(D)** Representative COX-2 detection by immunofluorescence and Western blot in fibroblast cultures. Mann-Whitney U-test was used for between-group comparisons and Wilcoxon test for paired comparisons. *, $P<0.05$ compared to non-stimulated cells; † $P<0.05$ compared to IL-1β-treated control-NM. AIA, aspirin-induced asthma; NM, nasal mucosa.



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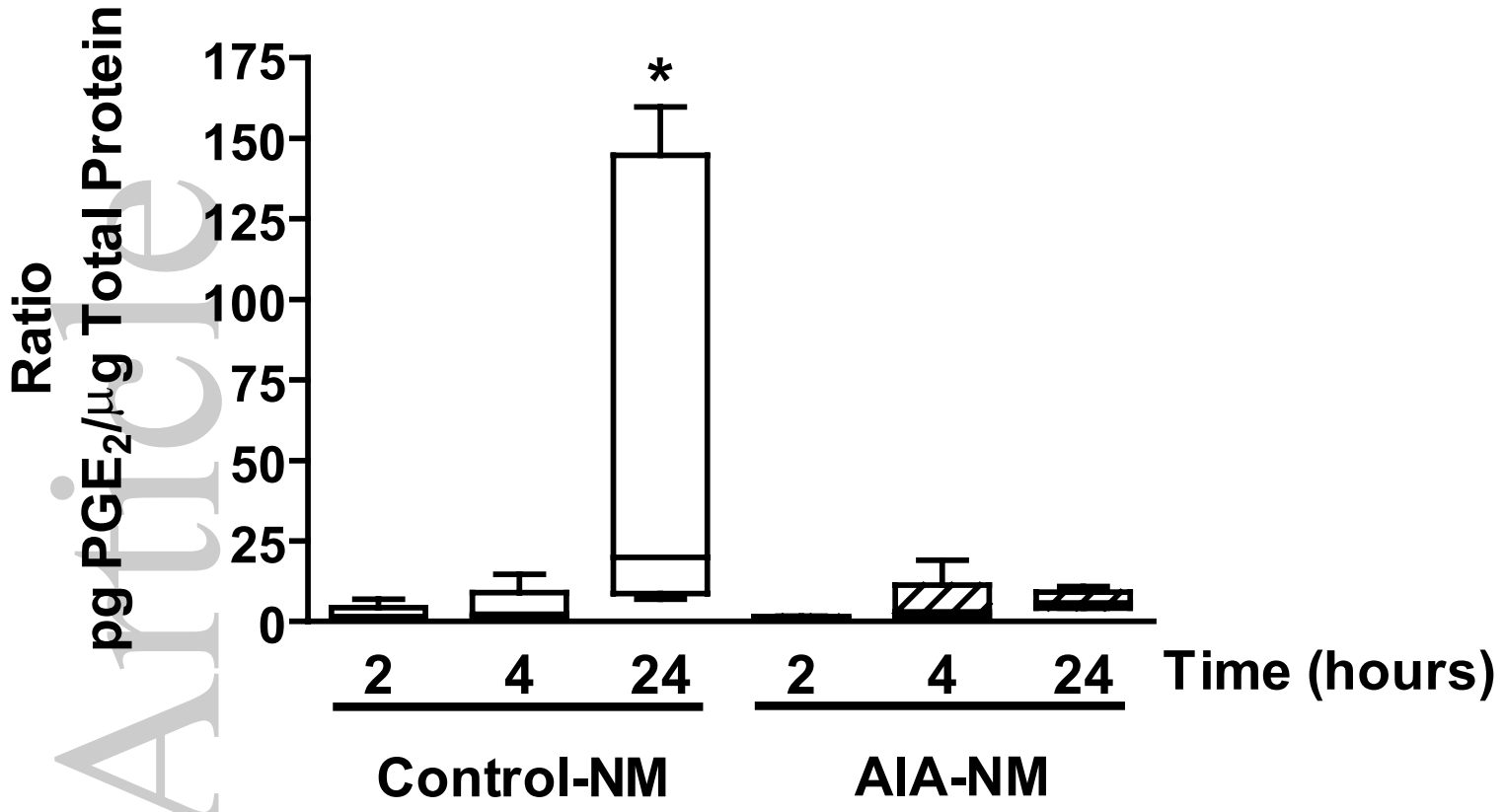

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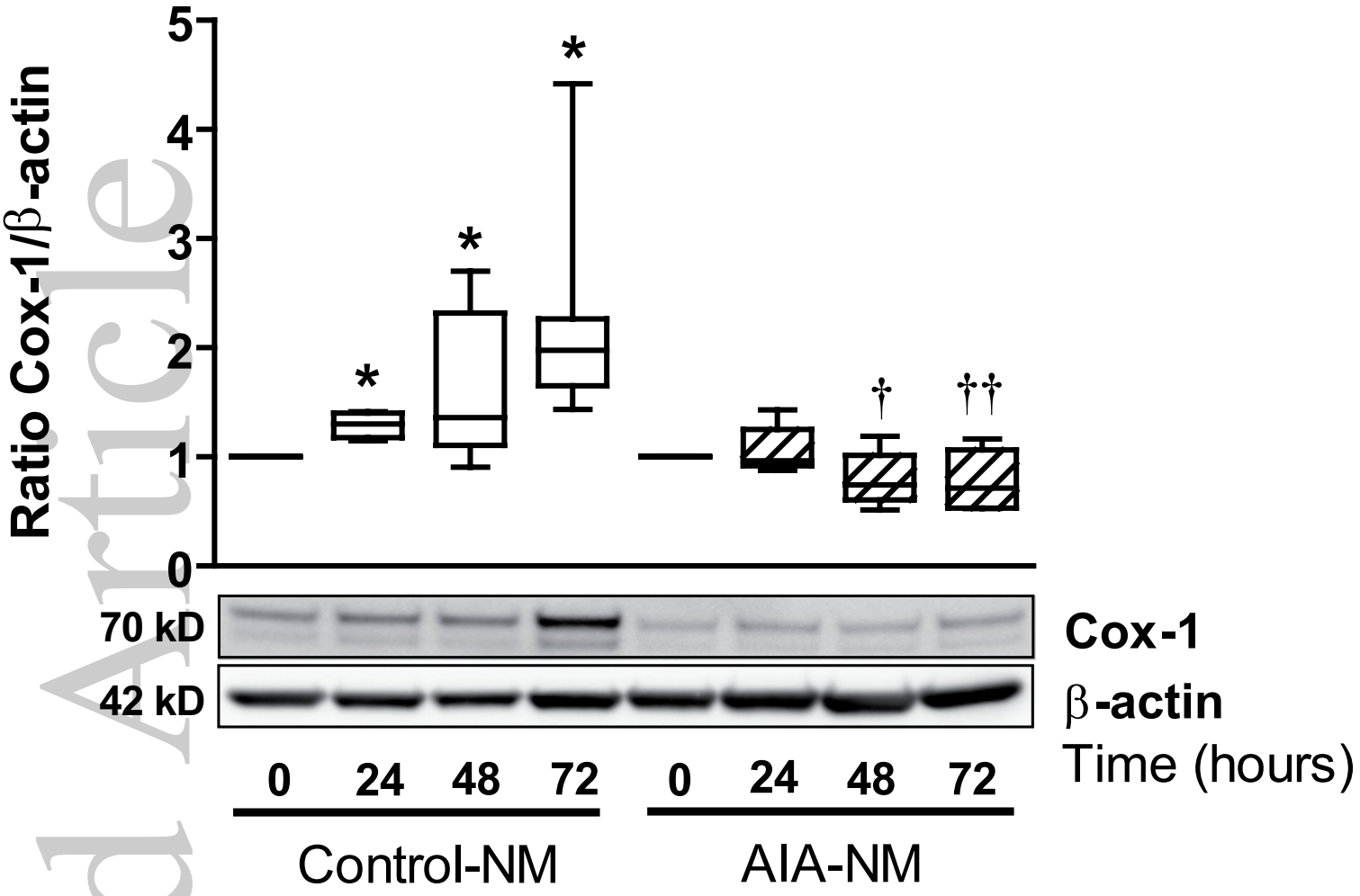
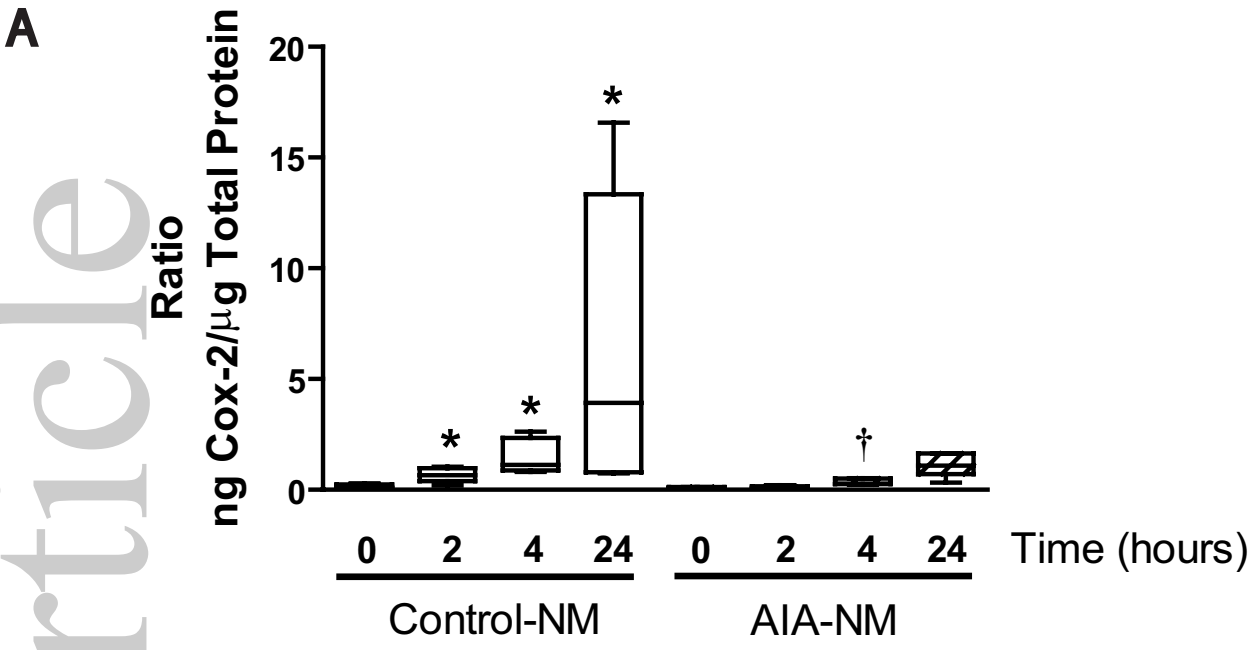
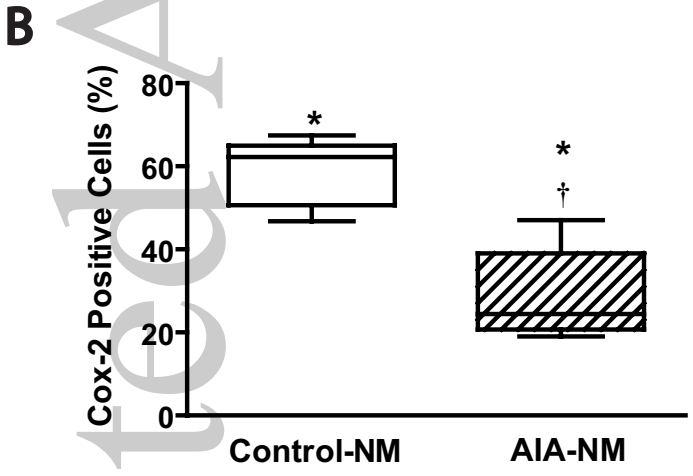
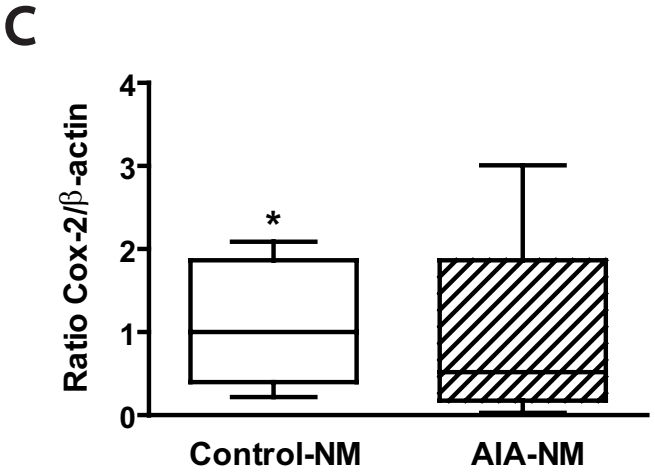


Figure 3 rev1.eps







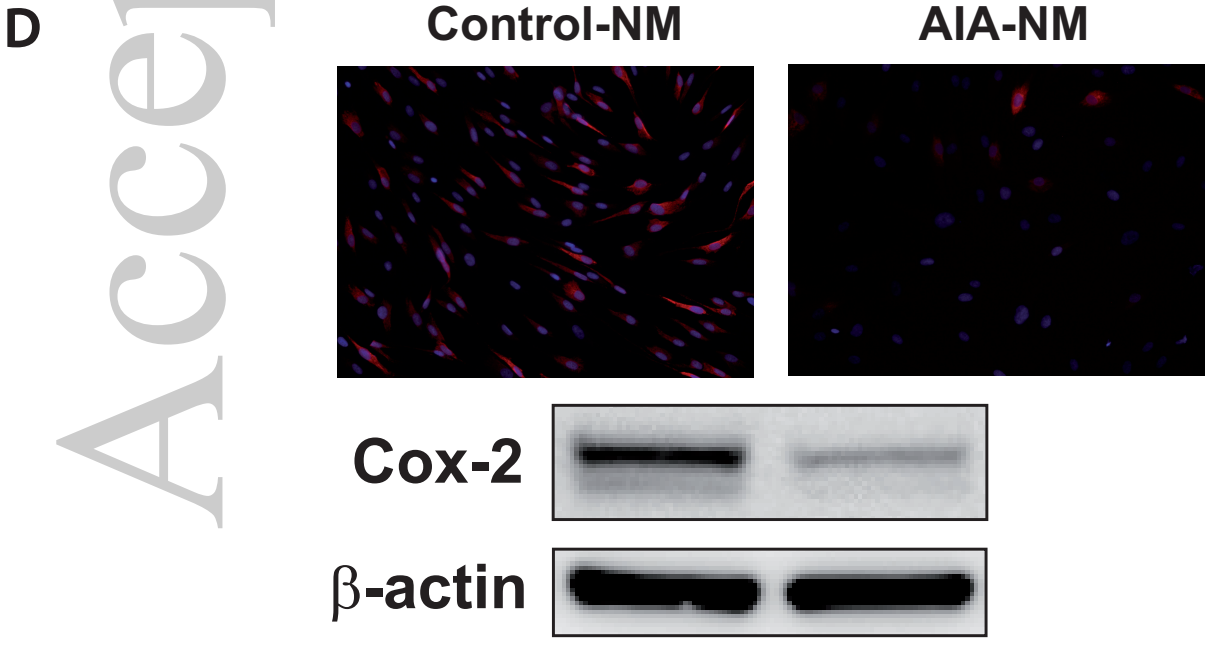


Figure 4 rev1.eps