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Glucosamine Hydrochloride Specifically Inhibits COX-2 by Preventing COX-2 *N*-Glycosylation and by Increasing COX-2 Protein Turnover in a Proteasome-dependent Manner*

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COX-2 and its products, including prostaglandin E_2, are involved in many inflammatory processes. Glucosamine (GS) is an amino monosaccharide and has been widely used for alternative regimen of (osteo)arthritis. However, the mechanism of action of GS on COX-2 expression remains unclear. Here we describe a new action mechanism of glucosamine hydrochloride (GS-HCl) to tackle endogenous and agonist-driven COX-2 at protein level. GS-HCl (but not GS sulfate, *N*-acetyl GS, or galactosamine HCl) resulted in a shift in the molecular mass of COX-2 from 72–74 to 66–70 kDa and concomitant inhibition of prostaglandin E_2 production in a concentration-dependent manner in interleukin (IL)-1β-treated A549 human lung epithelial cells. Remarkably, GS-HCl-mediated decrease in COX-2 molecular mass was associated with inhibition of COX-2 *N*-glycosylation during translation, as assessed by the effect of tunicamycin, the protein *N*-glycosylation inhibitor, or of cycloheximide, the translation inhibitor, on COX-2 modification. Specifically, the effect of low concentration of GS-HCl (1 mM) or of tunicamycin (0.1 μg/ml) to produce the aglycosylated COX-2 was rescued by the proteasomal inhibitor MG132 but not by the lysosomal or caspase inhibitors. However, the proteasomal inhibitors did not show an effect at 5 mM GS-HCl, which produced the aglycosylated or completely deglycosylated form of COX-2. Notably, GS-HCl (5 mM) also facilitated degradation of the higher molecular species of COX-2 in IL-1β-treated A549 cells that was retarded by MG132. GS-HCl (5 mM) was also able to decrease the molecular mass of endogenous and IL-1β- or tumor necrosis factor-α-driven COX-2 in different human cell lines, including Hep2 (bronchial) and H292 (laryngeal). However, GS-HCl did not affect COX-1 protein expression. These results demonstrate for the first time that GS-HCl inhibits COX-2 activity by preventing COX-2 co-translational *N*-glycosylation and by facilitating COX-2 protein turnover during translation in a proteasome-dependent manner.

Cyclooxygenase (COX),[3] also referred prostaglandin (PG) H synthase, is the rate-limiting enzyme in the biosynthesis of PGs and related eicosanoids from arachidonic acid metabolism (1). Physiologically, PGs are involved in inflammatory response, bone development, wound healing, and the reproductive system. If excessive, however, PGs play a pathogenic role in many chronic inflammatory and neoplastic diseases (1, 2).

In eukaryote cells, COX has two isoforms (1–3). COX-1 is constitutively expressed in most cells, and COX-1-derived PGs are involved in the maintenance of physiological functions. On the other hand, COX-2 is inducible by pro-inflammatory cytokines, tumor promoters, mitogenes, oncogenes, and growth factors in many types of cells, including monocytes, fibroblasts, and endothelial cells (1–5). Evidence that nonsteroidal anti-inflammatory drugs or compounds that target COX-2 lessen major inflammatory symptoms such as fever and pain suggests a role for COX-2 in inflammation (6). COX-2 expression is regulated at transcription, post-transcription, and translation. COX-2 transcription is induced by various exogenous stimuli that regulate intracellular signaling pathways that in turn modulate the activity of transcription factors and hence stimulate the COX-2 promoter (7). The cyclic AMP-responsive element, nuclear factor-interleukin 6, and NF-κB *cis*-acting elements were shown to be important for transcriptional COX-2 induction (8, 9). Stabilization and nuclear export of COX-2 mRNA at post-transcriptional levels are also necessary for maximal COX-2 induction (10–12). In addition, activities of MAPKs, including ERKs, p38 MAPK, and JNKs, were reported to be important for COX-2 expression (13, 14). COX-2 is an *N*-glycoprotein with four glycosylation sites (15, 16). Of interest, it has been previously shown that inhibition of COX-2 *N*-glycosy-

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[3] The abbreviations used are: COX, cyclooxygenase; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glucosamine; GS-HCl, glucosamine hydrochloride; IL, interleukin; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; PG, prostaglandin; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate; TNF, tumor necrosis factor; TN, tunicamycin; CHX, cycloheximide; Gal, galactosamine; CQ, chloroquine diphosphate salt; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; LEVD, *N*-acetyl-Leu-Glu-Val-Asp-CHO trifluoroacetate salt; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; p-, phospho-; RT, reverse transcription.

lation by site-directed mutagenesis or tunicamycin (TN), a protein *N*-glycosylation inhibitor, results in expression of COX-2 with the reduced molecular mass and activity (17), indicating the importance of this co-translational modification in COX-2 enzyme catalysis.

Glucosamine (GS) is an amino monosaccharide and has been widely used as an alternative regimen for rheumatoid arthritis or osteoarthritis. Recent *in vivo* studies have shown that GS salts, including GS sulfate or GS-HCl, have preventive actions on adjuvant arthritis in rats (18), possess the significant symptom-modifying effect on osteoarthritis in long term human clinical trials (19), and reduce equine cartilage degradation (20). Moreover, many recent *in vitro* studies have demonstrated that GS-HCl suppresses IL-1β-induced COX-2 expression by decreasing COX-2 transcript level in chondrocytes and synoviocytes (21), and that GS sulfate inhibits IL-1β-induced NF-κB activation in human osteoarthritic chondrocytes (22) and decreases TNF-α- and interferon-γ-induced ICAM-1 (intercellular adhesion molecule 1) expression at transcriptional level in human retinal pigment epithelial cells (23). From these, it is suggested that GS exerts its anti-inflammatory effect in part through transcriptional down-regulation of various genes involved in inflammation, cell adhesion, matrix degradation, and/or migration. However, the action mechanism by which GS affects expression and activity of COX-2 is not fully understood.

In this study, we evaluated the effects of different GS salts (GS-HCl, GS sulfate) or a GS derivative (*N*-acetyl GS) and galactosamine HCl (Gal-HCl), another hexosamine, on the expression of COX-2 and production of PGE_2 by IL-1β in A549 human lung epithelial cells. Here we demonstrate for the first time a new mechanism of GS-HCl to specifically inhibit endogenous and agonist-driven COX-2 at protein level.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, penicillin, and streptomycin were from Invitrogen. Fetal bovine serum was from Hyclone. GS-HCl, GS sulfate, *N*-acetyl GS, Gal-HCl, chloroquine diphosphate salt (CQ), calpain inhibitor I (ALLN), benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD), *N*-acetyl-Leu-Glu-Val-Asp-CHO trifluoroacetate salt (LEVD), TN, TNF-α, and actin antibody were from Sigma. NS398 and antibodies against COX-1 or COX-2 were from Cayman. MG132 was purchased from Calbiochem. An anti-*O*-linked GlcNAc monoclonal antibody RL2 was from Affinity BioReagents. Antibodies against anti-rabbit or mouse secondary horseradish peroxidase, ECL Western detection reagents, and PGE_2 enzyme-linked immunosorbent assay kit were from Amersham Biosciences. Actinomycin D and cycloheximide (CHX) were from Biomol. Bradford reagent was from Bio-Rad. Antibodies against phospho-ERKs (p-ERKs), phospho-JNKs (p-JNKs), and phospho-p38 MAPK (p-p38 MAPK) were from Cell Signaling Tech. Antibody against IκB-α was from Santa Cruz Biotechnology. PUGNAc, an inhibitor of *O*-GlcNAcase, was from CARBO GEN. IL-1β was from R & D Systems.

Cell Lines—Human cell lines used included A549 (lung), H292 (bronchial), and Hep2 (laryngeal). A549 and H292 cells were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hep2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Preparation of Whole Cell Lysates—Briefly, the cells were pretreated for 1 h with GS-HCl, GS sulfate, *N*-acetyl GS, Gal-HCl, NS398, TN, or PUGNAc and then exposed to IL-1β or TNF-α for 4 h. For the inhibition study, A549 cells were pretreated for 0.5 h with GS-HCl or TN. After removing the conditioned medium, the cells were once more exposed for 0.5 h to GS-HCl or TN plus respective inhibitor such as MG132, CQ, ALLN, Z-VAD, or LEVD. After pretreatment with drugs, the cells were then treated for 4 h with IL-1β and GS-HCl or TN plus respective inhibitor. After treatment, the cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF and lysed in a modified RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl). The supernatant was collected after centrifugation at 12,000 rpm for 20 min at 4 °C, and its protein concentration was then determined.

Western Blot Analysis—Equal amounts of proteins (40 μg/lane) were loaded onto 10% SDS-polyacrylamide gel. The separated proteins were transferred onto membrane (Millipore). The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and blocked in TBST containing 5% nonfat dried milk. The membrane was then incubated with antibodies of COX-1 (1:500), COX-2 (1:2,000), actin (1:10,000), p-JNKs (1:1000), p-ERKs (1:2000), p-p38 MAPK (1:2000), GRP78 (1:1,000), RL2 (1:1,000), or IκB-α (1:2,000). The membrane was then exposed to secondary antibodies coupled to horseradish peroxidase and developed in the ECL Western detection reagents.

PGE_2 Assay—PGE_2 was measured using the PGE_2 enzyme-linked immunosorbent assay kit (Amersham Biosciences). Briefly, 7×10^5 cells plated were pretreated with GS-HCl or NS-398, a COX-2-specific inhibitor, for 1 h and then treated with IL-1β for additional 4 h in the absence or presence of GS-HCl or NS-398. The conditioned medium was collected and subjected to PGE_2 assay in a 96-well plate according to the manufacturer's instructions. Principally, the product of this enzymatic reaction has a blue color that absorbs at 450 nm. The extent of color is inversely proportional to the amount of free PGE_2 present in the well during the incubation.

Reverse Transcription (RT)-PCR—Total RNA was isolated with the RNAzol-B (Tel-Test). Three micrograms of total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). Single-stranded cDNA was amplified by PCR with sequences of the following primers: COX-2 sense, 5′-TTGAAGACCAGGAGTACAGC-3′; COX-2 antisense, 5′-GGTACAGTTCCATGACATCG-3′; GAPDH sense, 5′-GGTGAAGGTCGGTGTGAACG-3′; and GAPDH antisense, 5′-GGTAGGAACACGGAAGGCCA-3′. The PCR

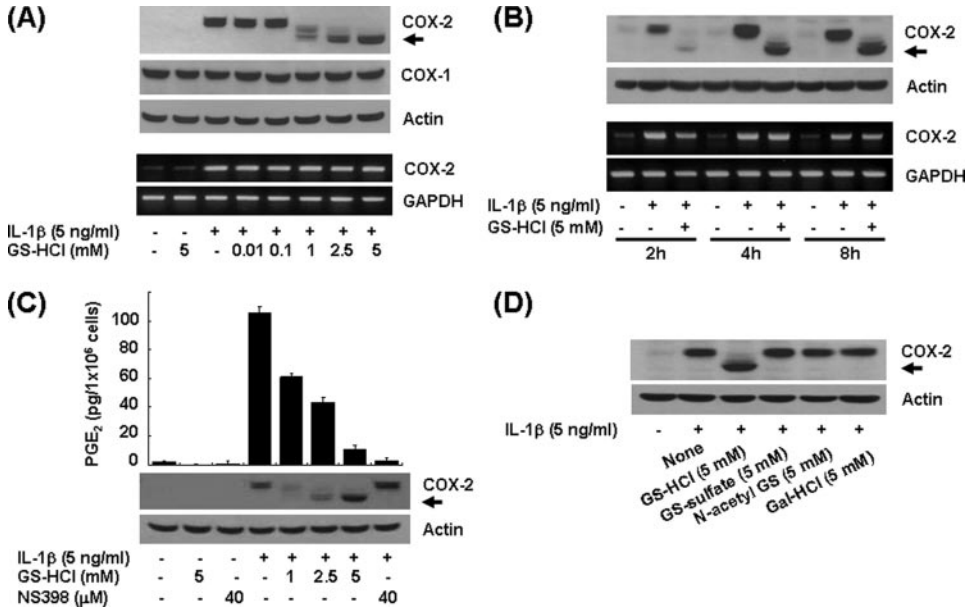

FIGURE 1. **GS-HCl specifically decreases the molecular mass and activity of COX-2 in IL-1β-treated A549 cells.** *A*, A549 cells were pretreated with the indicated concentrations of GS-HCl for 1 h. The cells were then exposed to IL-1β for 4 h in the absence or presence of GS-HCl. Whole cell lysates and total RNA were prepared and subjected to immunoblot analysis with antibodies specific for COX-1, COX-2, or actin and to RT-PCR for COX-2 or GAPDH mRNA, respectively. The *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment. *B*, A549 cells were pretreated with GS-HCl for 1 h. The cells were then treated with IL-1β for the indicated times in the absence or presence of GS-HCl. At each time point, whole cell lysates and total RNA were prepared and subjected to immunoblot analysis for COX-2 or actin protein and to RT-PCR for COX-2 or GAPDH mRNA, respectively. The *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment. *C*, A549 cells were pretreated with the indicated concentrations of GS-HCl or NS398 for 1 h. The cells were then exposed to IL-1β for 4 h in the absence or presence of GS-HCl or NS398. The conditioned culture medium was collected and subjected to enzyme-linked immunosorbent assay for PGE_2 production, and whole cell lysates were also prepared and analyzed by immunoblot analysis for COX-2 or actin. The *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment. *D*, A549 cells were pretreated with different GS salts (GS-HCl, GS sulfate), a GS derivative (*N*-acetyl GS), or Gal-HCl for 1 h. The cells were then exposed to IL-1β for 4 h without or with the indicated concentrations of GS-HCl, GS sulfate, *N*-acetyl, GS or Gal-HCl. Whole cell lysates were prepared and subjected to immunoblot analysis for COX-2 or actin. The *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment.

conditions applied were: COX-2, 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s; and GAPDH, 18 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. GAPDH was used as an internal control to evaluate the relative expression of COX-2.

Determination of COX-2 mRNA Stability—A549 cells were initially grown in the absence or presence of IL-1β for 4 h to highly induce endogenous COX-2 mRNA in A549 cells. The cells were then treated with IL-1β alone or IL-1β plus GS-HCl in the presence of actinomycin D, a transcription inhibitor, to block ongoing transcription for additional 1, 2, 4, or 8 h. At each time, total RNA was isolated and subjected to COX-2 or GAPDH RT-PCR to determine the amounts of each mRNA that remained in the cells.

Determination of COX-2 Protein Stability—A549 cells were initially grown in the absence or presence of IL-1β for 4 h to highly induce endogenous COX-2 protein in A549 cells. The cells were then treated with IL-1β alone or IL-1β plus GS-HCl for 2, 4, or 8 h in the absence or presence of CHX, a translation inhibitor. At each time, whole cell lysate was prepared and subjected to immunoblot analysis for COX-2 or actin to determine the amounts of each protein that remained in the cells.

Statistical Analysis—The results are expressed as the means ± S.E., and the significance of difference was determined by Student's *t* test. Differences were considered significant when the *p* value was less than 0.05.

RESULTS

Specific Effect of GS-HCl to Decrease the Molecular Mass and Activity of COX-2 in IL-1β-treated A549 Cells—The effect of different concentrations of GS-HCl on IL-1β-mediated expression of COX-2 in A549 cells was initially determined. As shown in Fig. 1*A*, IL-1β induced high COX-2 protein (*lane 3*) compared with control (*lane 1*). Treatment of cells with low concentrations of GS-HCl (0.01 and 0.1 mM) had no effect on expression of COX-2 protein induced by IL-1β (*lanes 4* and *5*). In contrast, treatment of cells with relatively high concentrations of GS-HCl (1–5 mM) led to the expression of COX-2 protein with an apparent molecular mass of ~66–70 kDa (*lanes 6–8*) as compared with the normally expressed 72–74-kDa protein (*lane 3*). Notably, treatment with GS-HCl at 5 mM led to strong accumulation of a distinct COX-2 with ~66 kDa (*lane 8*, marked with *arrow*). Of interest, A549 cells also expressed substantial levels of endogenous COX-1 protein that was not affected by IL-1β (Fig. 1*A*). Moreover, treatment with GS-HCl at concentrations used (0.01–5 mM) had no effect on the expression of COX-1 protein. Expression of actin protein was also not affected by GS-HCl, suggesting the specificity of GS-HCl to modify COX-2 at protein level. Remarkably, GS-HCl did not affect IL-1β-mediated expression of steady-state COX-2 mRNA (Fig. 1*A*). As shown in Fig. 1*B*, kinetic studies further demonstrated that pretreatment with GS-HCl (5 mM) did not affect newly synthesizing COX-2 mRNA expression (*lanes 1–9*) but led to the expression of COX-2 with mostly 66 kDa (marked with *arrow*) in A549 cells upon treatment with IL-1β in different times, indicating that GS-HCl does affect nascent COX-2 protein synthesis. Expression of either actin protein or GAPDH mRNA was also not affected by GS-HCl at times tested (Fig. 1*B*). To see whether GS-HCl-modified COX-2 is functional, the effect of GS-HCl on the production of PGE_2, a major and stable COX-2 metabolite, in IL-1β-treated A549 cells was next investigated. As shown in Fig. 1*C*, IL-1β increased PGE_2 production and COX-2 protein (*lane 4*) compared with control (*lane 1*), indicating that COX-2 induced by IL-1β is functional. NS398 was used as a positive control to assess the efficacy of GS-HCl to inhibit COX-2 activ-

ity. As anticipated, NS398 did not affect expression of COX-2 protein but almost completely inhibited PGE_2 production in IL-1β-treated A549 cells (*lane 8*), suggesting the efficacy of NS398 to inactivate COX-2. Importantly, there was a concentration-dependent trend in the decreased molecular mass of COX-2 and PGE_2 production in IL-1β-treated A549 cells following GS-HCl treatment (*lanes 5–7*). In particular, there was ~40 and 90% inhibition of PGE_2 production by GS-HCl at 1 and 5 mM, respectively. These results collectively suggest a correlation between the reduction of COX-2 molecular mass and the loss of COX-2 activity following GS-HCl treatment. Compared with GS-HCl (Fig. 1*D*, *lane 3*), however, GS sulfate, *N*-acetyl GS, or Gal-HCl did not influence the expression of COX-2 protein in IL-1β-treated A549 cells (Fig. 1*D*, *lanes 4–6*). Taken together, these results demonstrate that GS-HCl specifically inhibit COX-2 by decreasing its molecular mass and activity in IL-1β-treated A549 cells.

The Effect of GS-HCl to Decrease the Molecular Mass of COX-2 Is Unrelated with Protein O-Glycosylation and MAPKs Phosphorylation or NF-κB Pathway—The dynamic competition between protein glycosylation and phosphorylation has been reported (24). GS is shown to inhibit tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 and -2 in response to insulin signal that is associated with increased *O*-glycosylation of these proteins (25). Previously, it has been reported that prolonged incubation of PUGNAc, an inhibitor of *O*-GlcNAcase that cleaves *O*-linked glycan groups and not *N*-linked glycans from glycoproteins, results in increased *O*-glycosylation of cellular proteins (26). Using PUGNAc, we next asked whether the effect of GS-HCl on lowering the molecular mass of COX-2 is associated with the ability of GS-HCl to modulate protein *O*-glycosylation and/or phosphorylation in response to IL-1β. An anti-*O*-linked GlcNAc monoclonal antibody RL2 specific for *O*-glycosylated protein(s) was used. As shown in Fig. 2*A*, RL2 recognized many endogenous *O*-glycosylated proteins in A549 cells (*lane 1*). IL-1β alone did not affect the *O*-glycosylated proteins in A549 cells (*lane 4*). Apparently, GS-HCl (*lane 2*) or PUGNAc (*lane 3*) augmented or induced the *O*-glycosylated proteins in A549 cells (marked with *arrow*). Overall, PUGNAc enhanced or induced higher *O*-glycosylated proteins in IL-1β-treated A549 cells (*lane 6*) than GS-HCl (*lane 5*). Nevertheless, as shown in Fig. 2*B*, whereas GS-HCl led to decrease in the molecular mass of COX-2 in IL-1β-treated A549 cells (*lane 5*, marked with *arrow*), PUGNAc did not (*lane 6*). Notably, although low, A549 cells also expressed endogenous COX-2 protein (*lane 1*). Interestingly, GS-HCl was able to decrease the molecular mass of this endogenous COX-2 (*lane 2*), suggesting the ability of GS-HCl to modify both endogenous COX-2 and agonist (IL-1β)-driven COX-2. Actin level was not affected by treatment with GS-HCl or PUGNAc tested. It has been reported that IL-1β signals to increase phosphorylation (activation) of many signaling proteins, including ERKs, p38 MAPK, and JNKs, and their activations are necessary for the cytokine-induced COX-2 expression (27). This led us to test the effect of GS-HCl on protein phosphorylation in response to IL-1β. As shown in Fig. 3*A*, IL-1β stimulated or enhanced phosphorylation of ERK-1/2, JNK-1/2, and p38 MAPK in A549 cells (*lane 3*) without affecting the total protein level of ERK-1/2, JNK-1/2, or p38 MAPK (data not shown), suggesting the ability of IL-1β to increase phosphorylation of pre-existing ERK-1/2, JNK-1/2, or p38 MAPK. In IL-1β-treated A549 cells, GS-HCl had no effect on phosphorylation of ERK-1/2, JNK-1/2, and p38 MAPK (Fig. 3*A*, *lane 4*) but led to production of the low molecular mass of COX-2 (Fig. 3*B*, *lane 4*). Accordingly, IL-1β signals to degrade

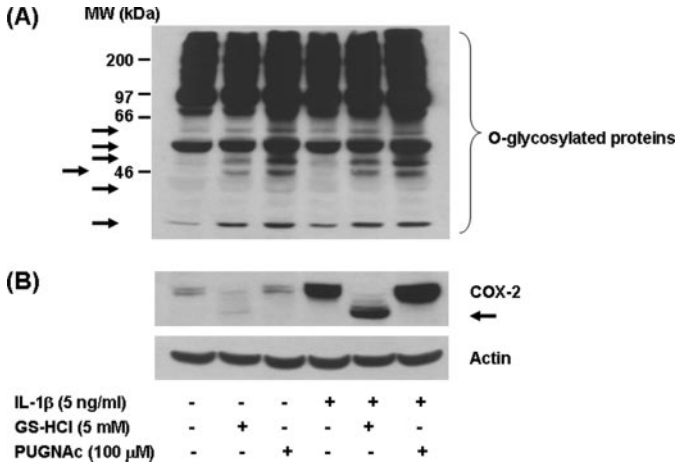

FIGURE 2. **Comparison of GS-HCl and PUGNAc on protein *O*-glycosylation and COX-2 expression in IL-1β-treated A549 cells.** A549 cells were pretreated with GS-HCl or PUGNAc, an inhibitor of GTFase that eventually leads to increased protein *O*-glycosylation, for 1 h. The cells were then treated with IL-1β for 4 h in the absence or presence of GS-HCl or PUGNAc. Whole cell lysates were prepared and analyzed by immunoblot analysis using an anti-*O*-linked GlcNAc monoclonal RL2 antibody that can detect *O*-glycosylated protein(s) (*A*) or antibodies for COX-2 or actin (*B*). In *B*, the *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment.

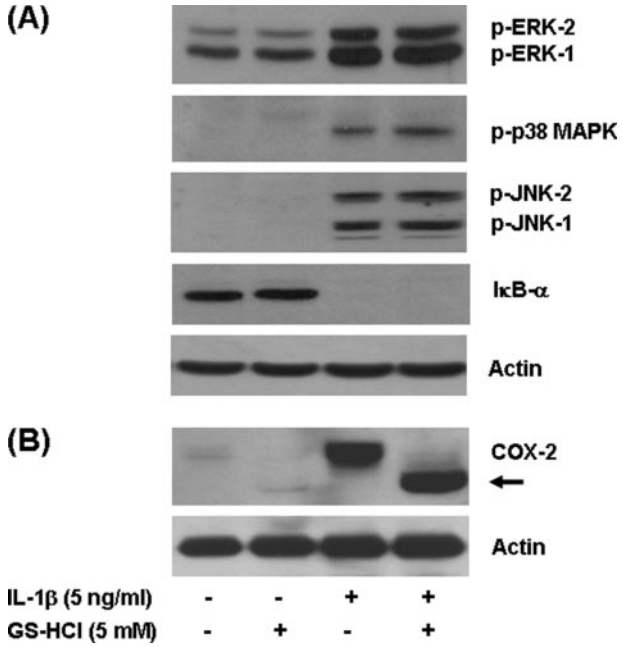

FIGURE 3. **Effect of GS-HCl on MAPK phosphorylation, IκB-α degradation, and COX-2 expression in IL-1β-treated A549 cells.** A549 cells were pretreated with GS-HCl for 1 h. The cells were then treated with IL-1β for 4 h in the absence or presence of GS-HCl. Whole cell lysates were prepared and analyzed by immunoblot analysis with antibodies specific for p-ERK-1/2, p-p38 MAPK, p-JNK-1/2, IκB-α or actin (*A*) or antibodies for COX-2 or actin (*B*). In *B*, the *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment.

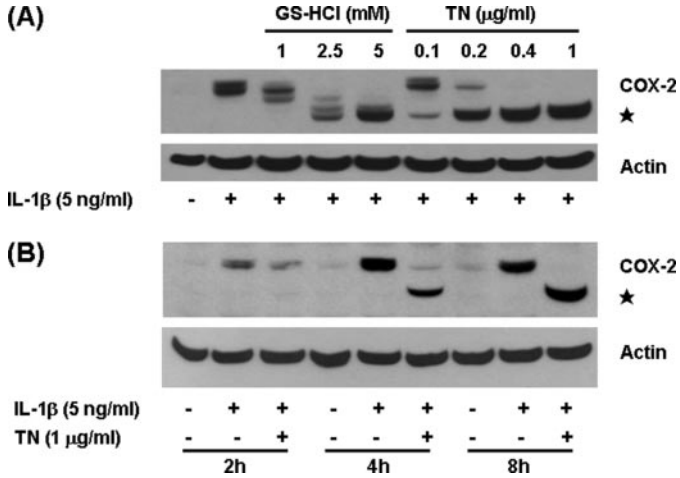

FIGURE 4. **Evidence for inhibition of COX-2 glycosylation by GS-HCl in IL-1β-treated A549 cells.** *A*, A549 cells were pretreated with the indicated concentrations of GS-HCl or TN, the protein *N*-glycosylation inhibitor, for 1 h. The cells were then treated with IL-1β for 4 h in the absence or presence of GS-HCl or TN. Whole cell lysates were prepared and analyzed by immunoblot analysis with antibodies for COX-2 or actin. The *star* indicates expression of the aglycosylated COX-2 with ~66 kDa following GS-HCl or TN treatment. *B*, A549 cells were pretreated with TN for 1 h. The cells were then treated with IL-1β for the indicated times in the absence or presence of TN. At each time point, whole cell lysates were prepared and subjected to immunoblot analysis for COX-2 or actin protein. The *star* indicates expression of the aglycosylated COX-2 with ~66 kDa following TN treatment.

IκB-α, a cytosolic inhibitory protein of NF-κB, and the resultantly activated NF-κB are involved in COX-2 transcriptional induction in A549 cells (8). GS sulfate is reported to inhibit IL-1β-induced NF-κB activation in chondrocytes (22). This led us to test whether GS-HCl affects IL-1β-mediated degradation of IκB-α in A549 cells. Distinctly, GS-HCl had no effect on IL-1β-mediated degradation of IκB-α (Fig. 3*A*, *lane 4*), suggesting that GS-HCl or GS sulfate may differentially regulate NF-κB activity in a cell-specific manner in response to IL-1β. Collectively, these results suggest that the effect of GS-HCl to decrease the molecular mass of COX-2 in IL-1β-treated A549 cells seems to be unrelated with protein *O*-glycosylation, MAPK phosphorylation, and/or NF-κB pathway.

GS-HCl-mediated Decrease in COX-2 Molecular Mass Is Associated with the Ability of GS-HCl to Inhibit COX-2 N-Glycosylation—COX-2 is an *N*-glycoprotein with 4 asparagine (Asn) sites, and if *N*-glycosylated, COX-2 is expressed in doublets, 72 and 74 kDa, with full enzymatic activities (15, 16). The mass difference between 72 and 74 kDa COX-2 is suggested to be due to variance of the degree of *N*-glycosylation on Asn^{580} (15). Interestingly, it has been recently shown that GS prevents *N*-glycosylation of certain proteins (29, 30), including glucose transporter 1 and a lipoprotein apoB-100. From these, we speculated that GS-HCl-modified COX-2 molecular mass and activity (Fig. 1*A*, *lanes 6–8*) might be associated with COX-2 *N*-glycosylation. TN is shown to inhibit COX-2 *N*-glycosylation, resulting in expression of the aglycosylated COX-2 with lowered molecular mass (~66 kDa) and activity (17). Thus, TN was used as a positive control to evaluate the efficacy of GS-HCl on COX-2 *N*-glycosylation. As clearly shown in Fig. 4*A*, TN treatment concentration-dependently inhibited COX-2 glycosylation, resulting in expression of the low molecular mass of COX-2 (mostly ~66 kDa) (*lanes 6–9*, marked with *star*), which is considered the fully agylcosylated COX-2, in IL-1β-treated A549 cells. Notably, the molecular mass of COX-2 mostly accumulated in IL-1β-treated A549 cells by either GS-HCl (5 mM) (*lane 5*) or TN (1 μg/ml) (*lane 9*) was nearly identical. Moreover, similar to the kinetic data of GS-HCl (Fig. 1*B*), pretreatment with TN (1 μg/ml) inhibited COX-2 glycosylation producing expression of aglycosylated COX-2 with 66 kDa (marked with *star*) in A549 cells following treatment with IL-1β in different times (Fig. 4*B*), suggesting that TN also targets glycosylation of newly synthesized COX-2. Altogether, these results convincingly suggest that GS-HCl or TN inhibits *N*-glycosylation of nascent COX-2 and subsequently produces the aglycosylated COX-2 in IL-1β-treated A549 cells.

Suppressive Effect of GS-HCl or TN on COX-2 N-Glycosylation by the Proteasome Inhibitor MG132—We have shown herein that GS-HCl concentration-dependently inhibited COX-2 glycosylation (Fig. 4*A*, *lanes 3–5*), concomitantly producing concentration-dependent hypoglycosylated forms of COX-2, which was nonfunctional (Fig. 1*C*, *lanes 5–7*). Nonfunctional or defective protein in cells is degraded via the proteasome-, lysosome-, or caspase-mediated proteolytic mechanisms. Using multiple pharmacological inhibitors such as the proteasome inhibitors (MG132, ALLN), the lysosome inhibitor (CQ), the pan-caspase inhibitor (Z-VAD), or the caspase-4 inhibitor (LEVD), we next challenged whether GS-HCl-modified COX-2 is associated with (non)proteolytic mechanisms. As shown in Fig. 5*A*, inhibition of COX-2 glycosylation by GS-HCl at 5 mM (*dashed arrow*) did not largely interfere with MG132, ALLN, CQ, Z-VAD, or LEVD. However, remarkably, appearance of the slightly hypoglycosylated COX-2 of 70 kDa by GS-HCl at 1 mM (*dashed arrow*) was inhibited by MG132 and ALLN, but not CQ, Z-VAD, and LEVD. Control experiments were also carried out to see whether the low molecular mass of (hypoglycosylated) COX-2 by GS-HCl at 1 mM can be produced by MG132 itself. As shown in Fig. 5*C* (*lane 7*), in the absence of GS-HCl, MG132 treatment failed to produce such a new low molecular mass of COX-2 in IL-1β-treated A549 cells; instead MG132 slightly inhibited IL-1β-induced expression of COX-2 protein. Profoundly, inhibition of COX-2 glycosylation by TN in IL-1β-treated A549 cells was also inhibited by MG132 (Fig. 5*D*, *lane 7*). These results collectively suggest involvement of the proteasomal pathway in production of the hypoglycosylayed COX-2 of 70 kDa by GS-HCl (1 mM) and of the aglycosylated COX-2 of 66 kDa by TN (0.1 μg/ml) in IL-1β-treated A549 cells.

GS-HCl Affects the Stability of COX-2 Protein in a Translation-dependent Manner—COX-2 expression is expected to be also influenced by COX-2 protein turnover. This led us to investigate the effect of GS-HCl on the stability of COX-2 protein in IL-1β-treated A549 cells. To do this, as seen in Fig. 6 (*A* and *B*), A549 cells were initially treated without (*lane 1*) or with IL-1β (*lanes 2–8*) for 4 h to highly increase COX-2 protein. After 4 h, a portion of cells (*lane 1* and *2*) was subjected to extract total cell lysates (as named at time 0 h). The rest of cells (*lanes 3–8*) were then exposed to IL-1β along without or with GS-HCl (5 mM) in the presence (Fig. 6*A*) or absence (Fig. 6*B*) of CHX for an additional 2, 4, or 8 h, following preparation of total

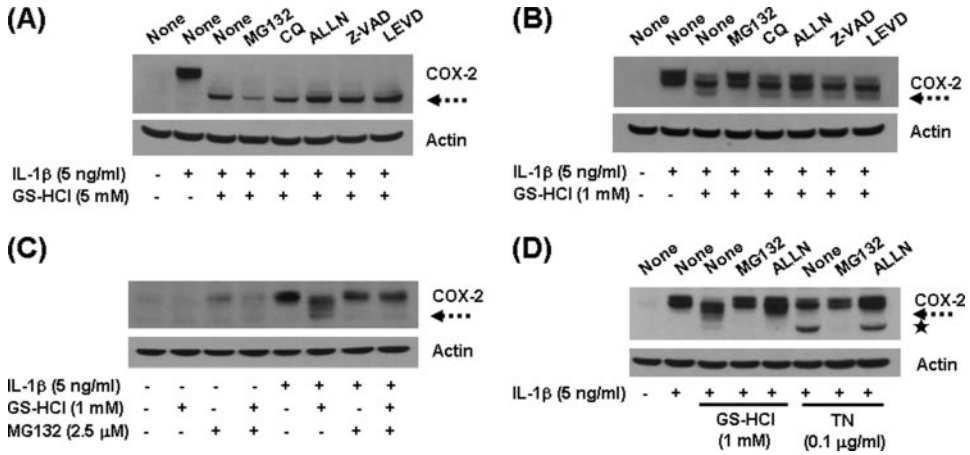

FIGURE 5. **Evidence for the proteasome-mediated inhibition of COX-2 glycosylation by GS-HCl or TN in IL-1β-treated A549 cells.** *A*, A549 cells were pretreated with GS-HCl with 5 mM in the absence or presence of MG132, ALLN, CQ, Z-VAD, or LEVD for 1 h. The cells were then treated with IL-1β for 4 h in the absence or presence of respective inhibitors. Whole cell lysates were prepared and analyzed by immunoblot analysis with antibodies for COX-2 or actin. The *dashed arrow* indicates expression of the aglycosylated COX-2 (66 kDa) following treatment with 5 mM GS-HCl. *B*, the same as *A* except treatment with 1 mM. The *dashed arrow* indicates expression of the partially hypoglycosylated COX-2 (70 kDa) following 1 mM GS-HCl. *C*, A549 cells were pretreated with GS-HCl or MG132 and both for 1 h. The cells were then treated with IL-1β for 4 h in the absence or presence of GS-HCl or MG132 and both. Whole cell lysates were prepared and analyzed by immunoblot analysis with antibodies for COX-2 or actin. The *dashed arrow* indicates expression of the partially unglycosylated COX-2 (66–72 kDa) following treatment with 1 mM GS-HCl. *D*, A549 cells were pretreated with GS-HCl or TN in the absence or presence of MG132 or ALLN for 1 h. The cells were then treated with IL-1β for 4 h along with GS-HCl or TN in the absence or presence of MG132 or ALLN. Whole cell lysates were prepared and analyzed by immunoblot analysis with antibodies for COX-2 or actin. The *dashed arrow* indicates expression of the partially hypoglycosylated COX-2 (70 kDa) following treatment with GS-HCl (1 mM). The *star* indicates expression of the aglycosylated COX-2 (66 kDa) following treatment with TN (0.1 μg/ml).

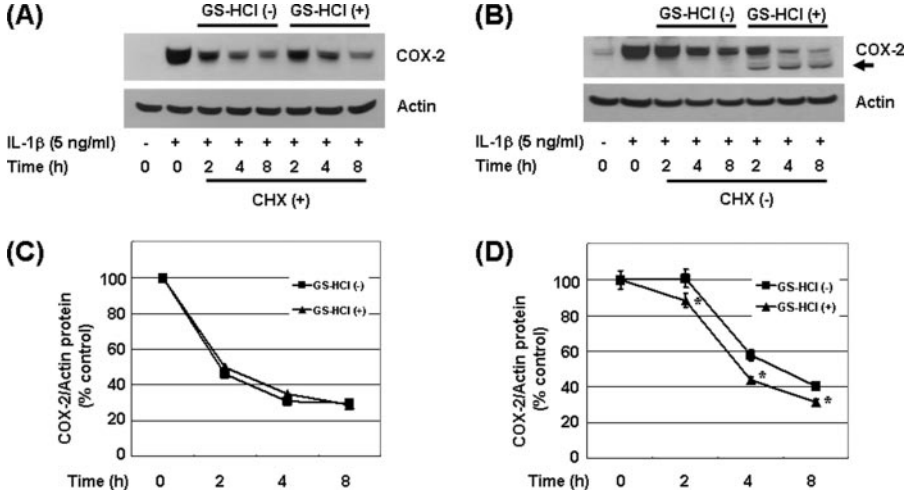

FIGURE 6. Differential effects of GS-HCl on inhibition of COX-2 glycosylation and COX-2 protein stability in the absence or presence of CHX. *A*, A549 cells were initially treated without (*lane 1*) or with (*lanes 2–8*) IL-1β for 4 h to highly induce the expression level of COX-2 protein and then exposed to IL-1β without (*lanes 3–5*) or with (*lanes 6–8*) GS-HCl in the presence (*lanes 2–8*) of CHX, a translational inhibitor, to block ongoing translation for the indicated times. At each time point, whole cell lysates were prepared and analyzed by immunoblot analysis using antibodies for COX-2 or actin to measure the amounts of COX-2 protein remained at respective times. *B*, A549 cells were treated with the same as *A* except for the absence of CHX. In *B*, the *arrow* indicates expression of the reduced molecular mass of COX-2 (66 kDa) following GS-HCl treatment. *C* and *D*, mean densitometric data for *A* and *B*, respectively, showing the level of COX-2 protein normalized to that of actin protein. *, $p < 0.05$ compared with the values of control (no GS-HCl) at the same time period. The number of experiments is three.

cell lysates at each time point. As shown in Fig. 6*A*, regardless of the absence or presence of GS-HCl, there was a similar pattern of time-dependent decrease in the amounts of COX-2 of 72–74 kDa in IL-1β-treated A549 cells, suggesting that if translation is inhibited, COX-2 protein is rapidly degraded (or destabilized), and GS-HCl does not affect COX-2 protein stability in IL-1β-treated A549 cells. Densitometric analysis for Fig. 6*A* clearly demonstrated no difference in the half-life ($t_{1/2}$) of COX-2, which was ~2 h, regardless of the absence or presence of GS-HCl, when translation was blocked with CHX (Fig. 6*C*). However, when translation was going (Fig. 6*B*), in the absence of GS-HCl, there was a relatively mild time-dependent decrease in the amounts of COX-2 of 72–74 kDa in IL-1β-treated A549 cells (*lanes 3–5*), which seemed to be due to newly synthesized COX-2 protein following additional IL-1β treatment. Remarkably, in the presence of GS-HCl, there was a drastic time-dependent decrease in the amounts of COX-2 of 72–74 kDa and concomitant production of COX-2 of 66 kDa (marked with *arrow*), presumably the aglycosylated COX-2, in IL-1β-treated A549 cells. The concomitantly produced COX-2 of 66 kDa following GS-HCl treatment during translation seemed to be associated with the ability of GS-HCl to inhibit *N*-glycosylation of newly synthesized COX-2 protein by additional IL-1β treatment, because there was no production of such lower molecular mass of COX-2 in the experimental conditions where IL-1β was not additionally added (data not shown). Thus, there might be no precursor-product relationship between the higher molecular species and the lower ones (Fig. 6*B*). Densitometric data for Fig. 6*B* further demonstrated that the $t_{1/2}$ of COX-2 in the absence and presence of GS-HCl was ~5.9 and 3.7 h, respectively (Fig. 6*D*). The $t_{1/2}$ of aglycosylated COX-2 was fairly stable (Fig. 6*B*). Altogether these data strongly suggest that in addition to preventing nascent COX-2 *N*-glycosylation, GS-HCl also facilitates degradation of higher molecular species of COX-2 during translation.

GS-HCl Appears to Accelerate the Proteasome-dependent Degradation of Higher Molecular Species of COX-2—The proteasomal pathway is involved in COX-2 protein turnover (31–34). This promptly led us to investigate whether the proteasomal pathway is associated with increased turnover of high molecular species of COX-2 by GS-HCl during translation. As

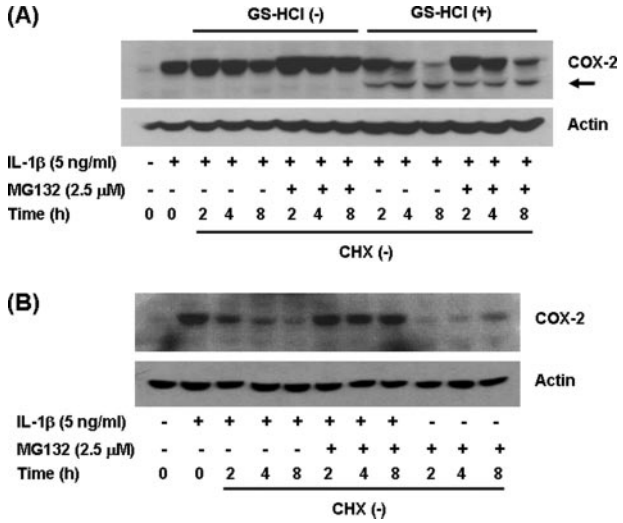

FIGURE 7. **Effects of MG132 on the stability of higher molecular COX-2 and the production and its protein stability of aglycosylated COX-2 in response to GS-HCl.** *A*, A549 cells were initially treated without (*lane 1*) or with IL-1β (*lanes 2–14*) for 4 h to highly induce levels of COX-2 protein, and then exposed to IL-1β (*lanes 2–14*) without (*lanes 3–8*) or with (*lanes 9–14*) GS-HCl (5 mM) in the absence (*lanes 3–5* and *9–11*) or presence (*lanes 6–8* and *12–14*) of MG132 for the indicated times in the absence of CHX. At each time point, whole cell lysates were prepared and analyzed by immunoblot analysis using antibodies for COX-2 or actin to measure the amounts of COX-2 protein remained at respective times. *B*, A549 cells were primarily treated without (*lanes 1* and *9–11*) or with IL-1β (*lanes 2–8*) for 4 h to highly induce levels of COX-2 protein. A549 cells were then grown in the absence (*lanes 1–5*) or presence (*lanes 6–11*) of MG132 to see the treatment effect of this proteasomal inhibitor on COX-2 protein stability (*lanes 6–8*) compared with control (no IL-1β, *lane 1*) and/or new COX-2 protein expression (*lanes 9–11*) compared with control (no MG132, *lanes 3–5*), for the indicated times in the absence of CHX. At each time point, whole cell lysates were prepared and analyzed by immunoblot analysis using antibodies for COX-2 or actin to measure the amounts of COX-2 protein remained at respective times.

shown in Fig. 7, in the absence of GS-HCl, MG132 treatment further enhanced the amounts of higher molecular species of COX-2 (*lanes 6–8*) when compared with those without MG132 (*lanes 3–5*) in IL-1β-treated A549 cells, suggesting that the proteasomal pathway mediates the turnover of COX-2 protein in IL-1β-treated A549 cells as long as translation is ongoing. Importantly, MG132 treatment effectively blocked GS-HCl-mediated decrease in the amounts of higher molecular species of COX-2 (*lanes 12–14*), suggesting involvement of the proteasomal pathway in GS-HCl-mediated COX-2 protein turnover during translation. However, production and stability of the aglycosylated COX-2 protein by GS-HCl in response to additional IL-1β treatment during translation (*lanes 9–11*) was not affected by MG132 (*lanes 12–14*, marked with *arrow*), suggesting that the events are proteasome-independent. It has been recently shown that inhibitors of the proteasome cannot only stabilize COX-2 protein, but they also induce new COX-2 expression (35, 36). This led us to determine whether the effect of MG132 on COX-2 protein turnover (Fig. 7*A*) was associated with the ability of MG132 to increase protein stability and/or new COX-2 synthesis. Control experiments were thus carried out in which A549 cells were primarily treated without (*lanes 1* and *9–11*) or with (*lanes 2–8*) IL-1β for 4 h. After removing the conditioned medium, the cells were then grown only in the absence (*lanes 3–5*) or presence (*lanes 6–11*) of MG132 without additional IL-1β treatment for the indicated times (Fig. 7*B*). The reason to exclude additional IL-1β treatment was to avoid new COX-2 protein expression by additional IL-1β treatment, which could allow us to better see the single treatment effect of MG132 on COX-2 protein expression and/or COX-2 protein stability. As expected, in the absence of MG132, COX-2 protein induced by IL-1β was time-dependently reduced (*lanes 3–5*), and MG132 treatment strongly blocked the decrease in the amounts of COX-2 protein (*lanes 6–8*). However, single treatment with MG132 for 2 or 4 h (*lane 9* or *10*) did not significantly induce expression of COX-2 protein in A549 cells. Interestingly, single treatment with MG132 for 8 h did induce low expression of COX-2 protein (*lane 11*). Considering such little or very low expression of COX-2 protein by single MG132 treatment in A549 cells, these results strongly suggest that the suppressive effect of MG132 on GS-HCl-mediated increase in turnover of higher molecular species of COX-2 (Fig. 7*A*) is through the ability of MG132 to increase the protein stability rather than its ability to induce new COX-2 protein. Collectively, these data demonstrate that in addition to preventing COX-2 *N*-glycosylation, GS-HCl also negatively affects COX-2 protein stability during translation particularly by facilitating the proteasomally dependent turnover of higher molecular species of COX-2.

No Effect of GS-HCl on COX-2 mRNA Stability—COX-2 expression is also regulated at the post-transcriptional level by mRNA degradation (10–12). Supporting it, increased post-transcriptional COX-2 mRNA stability is shown to contribute to IL-1β-induced COX-2 expression (37). However, no data are available as to the relationship between GS-HCl and COX-2 mRNA stability. This led us to test the effect of GS-HCl on COX-2 mRNA stability in IL-1β-treated A549 cells. The data of the actinomycin D chase experiments demonstrated that GS-HCl did not affect the stability of COX-2 mRNA in IL-1β-treated A549 cells (Fig. 8).

The Ability of GS-HCl to Decrease the Molecular Mass of Endogenous and Agonist-driven COX-2 in Different Cell Lines—To ascertain the specificity and generality of GS-HCl, the effect of GS-HCl (5 mM) on the expression of COX-1 and/or COX-2 protein in A549 cells treated with another COX-2 inducer such as TNF-α, another pro-inflammatory cytokine, was also investigated. As shown in Fig. 9*A*, the exposure of TNF-α into A549 cells induced COX-2 protein (*lane 2*). In TNF-α-treated A549 cells, GS-HCl specifically led to expression of the decreased molecular mass of COX-2, whereas GS sulfate, *N*-acetyl GS, or Gal-HCl did not influence expression of COX-2 protein, suggesting that GS-HCl is also able to inhibit COX-2 *N*-glycosylation in response to TNF-α signal. The effect of GS-HCl on the expression of COX-1 and/or COX-2 protein in different cell lines was also examined. As shown in Fig. 9*B*, IL-1β also slightly increased expression of COX-2 protein in Hep2 human bronchial epithelial cells (*lane 5*) compared with control (*lane 4*). Similar to the effect of GS-HCl to decrease the molecular mass of COX-2 in IL-1β-treated A549 cells (*lane 3*), GS-HCl could lead to expression of the lowered molecular mass of COX-2 in IL-1β-treated Hep2 cells (*lane 6*). Profoundly, H292 human laryngeal epithelial cells highly expressed endogenous COX-2 (*lane 7*), which was not further enhanced by IL-1β (*lane 8*), suggesting that there is no IL-1β receptor at the surface of the

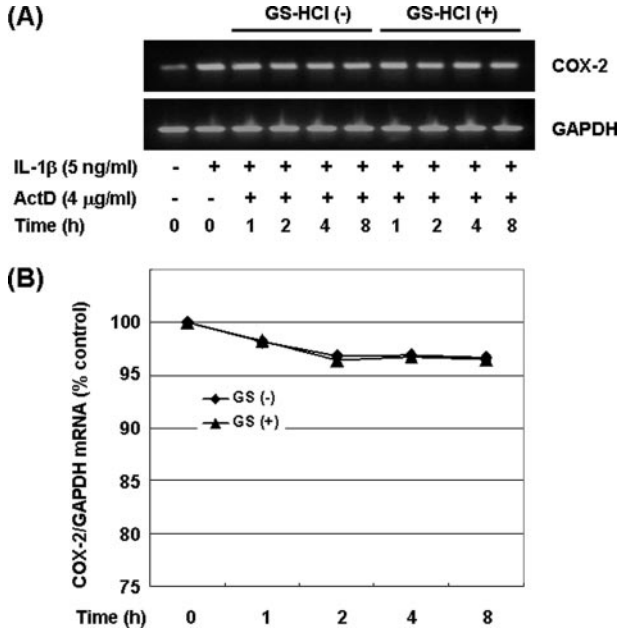

FIGURE 8. **No effect of GS-HCl on COX-2 mRNA stability.** *A*, A549 cells were initially treated without (*lane 1*) or with (*lanes 2–8*) IL-1β for 4 h to highly induce the expression level of COX-2 mRNA and then exposed to IL-1β without (*lanes 3–5*) or with (*lanes 6–8*) GS-HCl in the presence (*lanes 2–8*) of actinomycin D (*ActD*), a transcriptional inhibitor, to block ongoing transcription for the indicated times. At each time point, total RNA was isolated and used for COX-2 or GAPDH RT-PCR to measure the amounts of COX-2 mRNA remained in the cells. *B*, the graph shows COX-2 levels normalized to GAPDH mRNA levels. The data are from a representative experiment that was repeated twice.

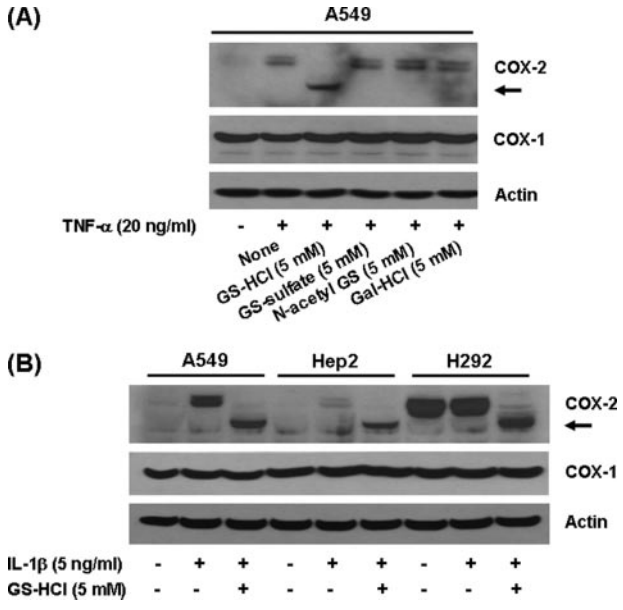

FIGURE 9. **Effect of GS-HCl on expression of COX-1 and COX-2 in A549 cells and other cell lines in response to IL-1β or TNF-α.** *A*, A549 cells were pretreated with GS-HCl, GS sulfate, *N*-acetyl GS, or Gal-HCl for 1 h. The cells were then exposed to TNF-α for 4 h in the absence or presence of GS-HCl, GS sulfate, *N*-acetyl GS, or Gal-HCl. Whole cell lysates were prepared and subjected to immunoblot analysis for COX-1, COX-2, or actin. *B*, other human cell lines, such as Hep2 (bronchial) or H292 (laryngeal) along with A549 (lung) were pretreated with GS-HCl for 1 h. Each cell line was then exposed to IL-1β for 4 h in the absence or presence of GS-HCl. Whole cell lysates were prepared and analyzed by immunoblot analysis for COX-1, COX-2, or actin. In *A* and *B*, the *arrow* indicates expression of the aglycosylated COX-2 (66 kDa) following GS-HCl treatment.

cells. Notably, GS-HCl strongly led to a decrease in the molecular mass of COX-2 that was endogenously expressed in H292 cells (*lane 9*). Supporting the specificity, GS-HCl did not affect COX-1 protein expressed in Hep2 or H292 cells (Fig. 8). These results collectively demonstrate the ability of GS-HCl to specifically decrease the molecular mass of endogenous and cytokine-driven COX-2 in different types of cells.

DISCUSSION

COX-2 and its products, including PGE_2, are involved in many inflammatory illnesses. Thus, any compound that inhibits COX-2 has the potential to be clinically useful against inflammatory diseases. In this study, we have evaluated the effect and action mechanism of different types of GS or other hexosamine on expression and activity of COX-2 in response to inflammatory cytokines. We here report for the first time that among GS salts or derivative and other hexosamine tested, GS-HCl specifically inhibits endogenous and cytokine-driven COX-2 expression at protein level via a mechanism associated with the proteasome-dependent down-regulation of COX-2 *N*-glycosylation and turnover.

GS is an amino monosaccharide and has been shown to possess anti-inflammatory efficacies against osteoarthritis and joint-degenerative disease in humans and animals (18–20). However, the action mechanism of this amino monosaccharide has not been fully elucidated. Previously, it has been reported that GS-HCl or GS sulfate inhibits IL-1β-induced COX-2 expression by transcriptional down-regulation in chondrocytes, and the down-regulation is in part associated with the ability of GS-HCl to inhibit NF-κB (22). In this study, however, we have demonstrated that GS-HCl does not affect the steady-state COX-2 mRNA level (Fig. 1*A*), the newly synthesizing COX-2 mRNA (Fig. 1*B*), the stability of COX-2 mRNA (Fig. 8), and the activation of NF-κB (Fig. 3*A*) in IL-1β-treated A549 cells. Instead, we have convincingly demonstrated that pretreatment of A549 cells with relatively high concentrations of GS-HCl (1–5 mM) leads to expression of COX-2 protein with an apparent molecular mass of ~66–70 kDa as compared with the normally expressed 72–74-kDa protein (Fig. 1*A*). Moreover, results of our kinetic studies showing that pretreatment with GS-HCl (5 mM) led to high accumulation of COX-2 of mostly 66 kDa in A549 cells upon the exposure of IL-1β at the indicated times (Fig. 1*B*) strongly suggest that GS-HCl may target nascent COX-2. Specifically, the present findings showing that treatment with GS sulfate, *N*-acetyl GS, or Gal-HCl does not affect expression of COX-2 protein by IL-1β in A549 cells (Fig. 1*D*) strongly suggest that GS-HCl has a unique ability to modify COX-2 protein in response to IL-1β signal. Importantly, through the present study, we have also demonstrated that GS-HCl concentration-dependently inhibits production of PGE_2 by IL-1β (Fig. 1*C*), further suggesting a strong correlation between a decrease in the molecular mass of COX-2 and a loss of COX-2 activity in response to GS-HCl. The differential effect of GS salts on COX-2 expression and NF-κB activation in response to IL-1β as shown in this study and in previous reports by others mentioned above might be

due to the different treatment times (4 h *versus* 24–48 h) and/or cell types (epithelial *versus* chondrocytes) applied.

Protein glycosylation affects the stability, activity, and/or cellular localization of protein. Although *O*-linked glycosylation is carried out in the Golgi, *N*-linked glycosylation occurs during co-translation process. Previously, it has been shown that treatment with GS leads to increased protein *O*-glycosylation (25). Consistent with it, in this study, we have shown that GS-HCl or PUGNAc enhances and/or induces *O*-glycosylation of some proteins in control or IL-1β-treated A549 cells (Fig. 2*A*). However, the present findings of the unique effect of GS-HCl, but not PUGNAc, on expression of the lowered molecular mass of COX-2 in IL-1β-treated A549 cells (Fig. 2*B*) suggest that the effect of GS-HCl on COX-2 modification is unrelated with the ability of GS-HCl to increase protein *O*-glycosylation. COX-2 is an *N*-glycoprotein and has been shown to possess four glycosylation sites within the structure (15, 16). Protein *N*-glycosylation is achieved by the action of oligosaccharyl transferase, an enzyme that adds *N*-glycan core, a glycocomplex consisting of nine mannoses, three glucoses, and two *N*-acetyl-GSs, to an Asn residue of newly synthesizing glycoprotein. Previously, it has been reported that the addition of *N*-glycan core gives the mass of ~2 kDa to the glycoprotein (38). Of interest, inhibition of protein *N*-glycosylation by GS has been addressed. For example, GS is shown to cause a shift in the mass of glucose transporter 1, an *N*-glycoprotein, from 45 to 38 kDa in L6 myocytes (29) and to induce defect of *N*-glycosylation of a lipoprotein apoB-100 in HepG2 cells (30). Further extending these previous reports, in this study, we have demonstrated the ability of GS-HCl to concentration-dependently shift the molecular mass of COX-2 from 72–74 to 66–70 kDa in IL-1β-treated A549 cells (Fig. 1*A*). TN is a well established inhibitor of protein *N*-glycosylation and truly has been demonstrated to inhibit *N*-glycosylation of COX-2, leading to expression of the aglycosylated COX-2 with decreased molecular mass and activity (17). The present findings from experiments with TN, which shows a nearly identical effect on COX-2 protein modification by GS-HCl (Fig. 4), provide convincing evidence that the low molecular species of COX-2 by GS-HCl are hypoglycosylated proteins. Additionally, the reduced catalytic activity of the low molecular mass species of COX-2 produced following GS-HCl treatment (Fig. 1*C*) is consistent with previous *in vitro* experiments that demonstrate a requirement for the translationally coupled glycosylation of COX-2 to produce active COX-2 (17). Thus, these results strongly suggest that GS-HCl decreases the molecular mass and activity of COX-2 by preventing the co-translational *N*-glycosylation of the enzyme.

In this study, we have demonstrated that GS-HCl affects the stability of COX-2 protein in a translation-dependent manner. Previously, it was shown that the $t_{1/2}$ of COX-2 protein in murine fibroblasts was ~2 h (31). Consistent with it, we have demonstrated herein that if translation is blocked, in the absence of GS-HCl, COX-2 induced by IL-1β in A549 cells is rapidly degraded with a $t_{1/2}$ of ~2.0 h (Fig. 6, *A* and *C*). The rapid COX-2 turnover was not altered by the addition of GS-HCl (Fig. 6, *A* and *C*), suggesting that GS-HCl does not affect COX-2 protein stability in A549 cells as long as translation is blocked. However, we have demonstrated that as long as translation is ongoing, in the absence of GS-HCl, the turnover of COX-2 is retarded with a $t_{1/2}$ of ~5.9 h (Fig. 6, *B* and *D*), which seems to be attributable to newly synthesized COX-2 protein by additional treatment with IL-1β during translation. Profoundly, as long as translation is ongoing, we have shown that GS-HCl treatment leads to rapid disappearance of the higher molecular species of COX-2 and concomitant production of the low molecular species of stable aglycosylated COX-2 in IL-1β-treated A549 cells (Fig. 6*B*). Considering that the effect of GS-HCl to tackle nascent COX-2 protein (Fig. 1*A*) and blocking COX-2 protein synthesis with CHX prevents the decrease in COX-2 molecular mass attributed to GS-HCl in IL-1β-treated A549 cells (Fig. 6*A*), the stable aglycosylated COX-2 seen by GS-HCl treatment in IL-1β-treated A549 cells (Fig. 6*B*) appears to be derived from the newly synthesized COX-2 by additional IL-1β treatment during translation. Densitometric data have demonstrated that GS-HCl treatment increases the turnover of COX-2 with a $t_{1/2}$ of 3.7 h (Fig. 6*D*). Collectively these findings strongly suggest that in addition to preventing COX-2 *N*-glycosylation, GS-HCl facilitates the turnover of COX-2 as long as translation is ongoing. To our knowledge, it also seems to be the first addressing GS-HCl regulation of COX-2 protein turnover.

The presence or accumulation of nonfunctional or defective protein, including misfolded or aglycosylated one, may be noneconomic and harmful to cells. Thus, cells must maneuver cellular protein degradative systems, including the proteasome-, lysosome-, and/or caspase-mediated proteolytic pathways, to dispose them. Previously, it has been shown that GS treatment causes defect of *N*-glycosylation of apoB-100 protein, which results in post-ER degradation via nonproteasomal pathways in HepG2 cells (30). Specifically, in this study, we have shown that in IL-1β-treated A549 cells, the proteasomal inhibitor MG132 (but not the lysosomal or caspase inhibitors) effectively inhibits GS-HCl (1 mM)-mediated expression of the hypoglycosylated COX-2 (Fig. 5*B*), although MG132 does not show an effect at 5 mM GS-HCl, which produces the aglycosylated or completely deglycosylated form of COX-2 (Fig. 5*A*). It is speculative that the proteasome inhibitor may block synthesis of the new low molecular mass of COX-2 in response to GS-HCl at low concentration (1 mM). However, the control experiment with MG132 itself, which shows no synthesis of the new low molecular mass of COX-2 (Fig. 5*C*), bolsters that it is not the case. Additionally, we have demonstrated that MG132 also inhibits TN (0.1 μg/ml)-mediated expression of the aglycosylated COX-2 in IL-1β-treated A549 cells (Fig. 5*D*). These findings strongly suggest that the 26 S proteasomal pathway participates in preventing COX-2 *N*-glycosylation by GS-HCl or TN in IL-1β-treated A549 cells. Recently, it has been shown that the 26 S proteasomal pathway contributes to COX-2 protein turnover (31–34). In this study, we have demonstrated that the turnover of higher molecular species of COX-2 in IL-1β-treated A549 cells is retarded by MG132

(Fig. 7*B*), suggesting that the 26 S proteasomal pathway mediates degradation of the higher molecular species of COX-2 in IL-1β-treated A549 cells. Importantly, the present findings showing that MG132 effectively blocks GS-HCl-mediated increase in the turnover of higher molecular species of COX-2 (Fig. 7*A*) suggest that GS-HCl facilitates COX-2 protein turnover via the proteasomal pathway in IL-1β-treated A549 cells. Recently, it has been shown that inhibitors of the proteasome not only stabilize COX-2 protein but also induce new COX-2 expression (35, 36). However, the present findings showing that there is no significant effect of treatment with MG132 alone on expression of COX-2 protein in A549 cells suggest that the inhibitory effect of MG132 on GS-HCl-mediated enhancement of turnover of the higher molecular species of COX-2 (Fig. 7*A*) is closely associated with the ability of MG132 to increase COX-2 protein stability rather than its ability to induce new COX-2 expression. Nevertheless, although there is weak evidence, these present data collectively suggest that GS-HCl enhances COX-2 protein turnover in a proteasome-dependent manner during translation, although it will be necessary in the future to perform ^{35}S pulse-chase experiments to convincingly demonstrate it.

Whether GS-HCl inhibits COX-2 *N*-glycosylation directly or indirectly and/or which cellular factors facilitate this event remain unclear at this moment. *N*-Glycanase is an enzyme responsible for removing *N*-glycans from glycoproteins and has been shown to be involved in protein degradation by interacting with the proteasomal pathway (28). Considering these, we speculate that a possible mechanism of the proteasome-mediated inhibition of COX-2 *N*-glycosylation by GS-HCl may be that GS-HCl may affect expression and/or activity of oligosaccharyl transferase and/or *N*-glycanase by modulating the proteasomal pathway. In view of this, it will be interesting to see in the future whether knock-down or overexpression of these enzymes will affect GS-HCl-mediated COX-2 modification in conjunction with the proteasomal pathway. It is also interesting to note that the proteasomal pathway is not effective in preventing COX-2 *N*-glycosylation by GS-HCl at high concentration (5 mM), suggesting that the proteasome-dependent inhibition of COX-2 *N*-glycosylation may be an event occurring only at low concentrations of GS-HCl.

Another interesting finding in this study was the ability of GS-HCl to tackle COX-2 but not COX-1. In this study, we have demonstrated that A549 cells endogenously express both COX-1 and COX-2, but GS-HCl decreases the molecular mass of COX-2 but not COX-1 (Fig. 1, *A* and *D*), suggesting that GS-HCl specifically targets COX-2 in cells co-expressing both COX-1 and COX-2. By performing additional cell culture works, we have apparently shown the ability of GS-HCl to decrease the molecular mass of not only endogenous but also IL-1β or TNF-α-driven COX-2 in different human cell lines, including H292 (laryngeal) or Hep2 (bronchial), without influencing COX-1 (Fig. 8). Cell proliferation assay using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] or cell count analysis used to determine the number of viable cells in proliferation suggest no cytotoxicity in all cell lines used in this study after treatment with GS-HCl at 1–5 mM concentrations in which COX-2 protein was modifiable (data not shown).

In summary, we report for the first time that GS-HCl inhibits COX-2 by preventing COX-2 co-translational *N*-glycosylation and by facilitating COX-2 protein turnover during translation in a proteasome-dependent manner. The findings presented herein may provide a potential explanation for the clinical effect of GS supplements, particularly GS-HCl herein, which are purported to have an anti-inflammatory activity.

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Enzyme Catalysis and Regulation:

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