

Transcriptional and translational regulation of COX-2 expression by cadmium in C6 glioma cells

YU-KYOUNG PARK, HUA HONG and BYEONG-CHURL JANG

Department of Medical Genetic Engineering, School of Medicine, Keimyung University,
Dalseo-gu, Daegu 704-701, Republic of Korea

Received April 6, 2012; Accepted June 11, 2012

DOI: 10.3892/ijmm.2012.1052

Abstract. High exposure to cadmium is a risk factor for many neuronal diseases. Overexpression of cyclooxygenase (COX)-2 is linked to many neuroinflammatory and neoplastic diseases. We, herein, investigated the effect of cadmium on the expression of COX-2 in C6 rat glioma cells. Treatment with cadmium sulfate (cadmium) increased the expression of COX-2 mRNA. Remarkably, cadmium treatment further increased expression of not only the N-glycosylated COX-2 protein of 72 kDa but also the unglycosylated COX-2 of 66 kDa, as assessed by the unglycosylated COX-2 induced by tunicamycin or glucosamine, known inhibitors of COX-2 N-glycosylation. Of note, when translation was blocked in the presence of cycloheximide (CHX), levels of both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium rapidly declined but the decline was prevented by MG132, a 26S proteasomal inhibitor. However, in the absence of CHX, cadmium induced and maintained expression of the unglycosylated COX-2 proteins. Pharmacological inhibition studies importantly demonstrated that the cadmium-mediated COX-2 transcriptional upregulation in C6 cells was not shown by exogenous glutathione (GSH) supplementation or treatment with inhibitors of extracellular signal-regulated protein kinase-1/2 (ERK-1/2), p38 MAPK and c-Jun N-terminal protein kinase-1/2 (JNK-1/2), respectively. Expression of COX-2 was not noted in C6 cells exposed to other heavy metals (cobalt or manganese). These results demonstrate that cadmium specifically induces expression of COX-2 through both transcriptional and co-translational (N-glycosylation) regulation in C6 cells in which the cadmium-induced COX-2 transcriptional upregulation is closely related to oxidative stress-dependent activation of the family of MAPKs and the cadmium-induced expression

of both N-glycosylated and unglycosylated COX-2 proteins is proteasome- and translation-dependent.

Introduction

Cadmium is a toxic heavy metal and an environmental pollutant. Evidence suggests that cadmium, due to its long half-life, can accumulate in the body, mostly in the brain, kidney, and lung (1,2). Importantly, many *in vitro* and *in vivo* studies have demonstrated that acute or chronic cadmium exposure causes toxic and carcinogenic effects on various tissues, including brain, liver, kidney, lung, prostate and testis (3-6). However, the molecular and cellular mechanisms underlying cadmium-mediated toxic and tumorigenic effects on tissues still remain elusive.

Cyclooxygenase (COX), also referred to as prostaglandin (PG) H synthase, is the rate-limiting enzyme in the biosynthesis of PGs and related eicosanoids from arachidonic acid metabolism (7). Physiologically, levels of PGs are involved in the inflammatory response, bone development, wound healing, and reproductive system. However, excessive PGs are linked to many inflammatory and neoplastic diseases. In eukaryotic cells, COX is expressed in two isoforms, COX-1 and COX-2 (8,9). While COX-1 is constitutively expressed in most cells and PGs produced by COX-1 are involved in the maintenance of physiological functions, COX-2 is inducible by pro-inflammatory cytokines, tumor promoters, mitogenes, and growth factors in a variety of cell types, including monocytes, fibroblasts and endothelial cells (8-10). Evidence that non-steroidal anti-inflammatory drugs or compounds that inhibit COX-2 lessen major inflammatory symptoms including fever and pain suggests a role of COX-2 in inflammation (11). Notably, COX-2 expression has been shown to be increased in glia of rats with traumatic brain injury (12) and of a mouse model of sporadic amyotrophic lateral sclerosis (13), suggesting a role of glial COX-2 expression in brain injury or neurodegenerative disease. Moreover, of further interest, it has been demonstrated that COX-2 is upregulated in the majority of high-grade human gliomas, and blockage of COX-2 activity by NS-398, a selective COX-2 inhibitor, inhibits growth and induces apoptosis of multiple glioma cells (14). However, the regulation of COX-2 expression in glioma is not well understood.

Expression of COX-2 is regulated at levels of transcription, post-transcription and translation. Transcription of COX-2 is induced in cells following treatment with exogenous stimuli,

Correspondence to: Professor Byeong-Churl Jang, Department of Medical Genetic Engineering, School of Medicine, Keimyung University, 1095 Dalgubeol-daero, Dalseo-gu, Daegu 704-701, Republic of Korea
E-mail: jangbc12@kmu.ac.kr

Key words: cadmium, cyclooxygenase-2, N-glycosylation, glutathione, MAPKs, C6 glioma cells

which is accompanied by activation of intracellular signaling pathways that in turn modulate activity of transcription factors (TFs) and hence stimulate the COX-2 promoter (15). Studies have demonstrated the presence of multiple cis-acting elements, including nuclear factor- κ B (NF- κ B), activation of transcription factor-2 (ATF-2), and activator protein 1 (AP-1), within the COX-2 promoter, which are bound by specific TFs, and their importance for COX-2 transcriptional induction (16,17). Evidence also strongly suggests the post-transcriptional regulation of COX-2 at levels of message stability and RNA nuclear export and COX-2 translational regulation by the protein turnover are critical for maximal COX-2 induction (18-20). It is also documented that COX-2 protein N-glycosylation during translation is critical for the protein stability, localization, and activity (21-23). Moreover, there is evidence that activities of a variety of cellular signaling proteins, such as extracellular signal-regulated protein kinase-1/2 (ERK-1/2), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal protein kinase-1/2 (JNK-1/2), and protein kinase B, are linked to upregulation of COX-2 expression by influencing levels of COX-2 transcription, post-transcription, and/or translation (24,25). However, little is known concerning the regulation of COX-2 expression by cadmium in glioma cells.

In this study, we investigated the effect of cadmium on COX-2 expression in C6 rat glioma cells and determined possible molecular and cellular mechanisms involved.

Materials and methods

Materials. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from WelGENE (Daegu, Korea). Antibodies of phospho-ERK-1/2 (p-ERK-1/2), total ERK-1/2 (T-ERK-1/2), phospho-JNK-1/2 (p-JNK-1/2), total JNK-1/2 (T-JNK-1/2), phospho-p38 MAPK (p-p38 MAPK), total p38 MAPK (T-p38 MAPK) and phospho-ATF-2 (p-ATF-2) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-COX-2 rabbit polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-rabbit or mouse secondary horseradish peroxidase antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MG132, N-acetyl cysteine (NAC), SP600125, and proteinase inhibitor cocktail (100X) were purchased from Calbiochem (Madison, WI, USA). SB203580, PD98059 and LY294002 were purchased from BioMol (Plymouth Meeting, PA, USA). RNazol-B reagent was purchased from Tel-Test, Inc. (Houston, TX, USA). Enzyme-linked chemiluminescence (ECL) Western detection reagents were purchased from Thermo Scientific (Waltham, MA, USA). Nitrocellulose membrane was purchased from Millipore (Rockford, IL, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Other reagents, including anti-actin mouse monoclonal antibody, cadmium (CdSO_4), glucosamine hydrochloride (GS-HCl), tunicamycin (TN), cycloheximide (CHX), glutathione (GSH), vitamin E (VE), cobalt chloride (CoCl_2) and manganese (MnCl_2) were purchased from Sigma (St. Louis, MO, USA).

Cell culture. C6 rat glioma cells were maintained at 37°C in a humidified condition of 95% air and 5% CO_2 in DMEM

supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin.

Preparation of whole cell lysates. After treatments at the indicated times or conditions, C6 cells were washed with ice-cold phosphate-buffered saline (PBS) supplemented with 1 mM Na_3VO_4 and 1 mM NaF, and lysed in modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was collected and the protein concentration was determined with Bradford reagent (Bio-Rad, Mississauga, ON, USA) using bovine serum albumin as the standard.

Western blot analysis. Equal amounts of protein (40 $\mu\text{g/lane}$) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was washed with Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST supplemented with 5% non-fat dried milk. The membrane was further incubated with primary antibodies of COX-2 (1:2,000), p-JNK-1/2 (1:2,000), T-JNK-1/2 (1:2,000), p-ERK-1/2 (1:2,000), T-ERK-1/2 (1:2,000), p-p38 MAPK (1:2,000), T-p38 MAPK (1:2,000), p-ATF-2 (1:2,000) or actin (1:10,000). The membrane was subsequently incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed in ECL Western detection reagents.

Reverse transcription-polymerase chain reaction (RT-PCR). After treatments at the indicated times or conditions, total cellular RNA was then isolated from the conditioned cells using RNazol B reagent according to manual instructions provided by the manufacturer. Five micrograms of total-RNA was reverse transcribed using 8 μl of M-MLV RT 5X buffer, 3 μl of 10 mM dNTPs, 0.45 μl of 10,000 units RNase inhibitor, 0.3 μl of 50,000 units M-MLV reverse transcriptase (Promega, Madison, WI), and 1.5 μl of 50 pM oligo(dt) (Bioneer, Chungbuk, Korea) in 40 μl volume. Single stranded cDNA was then amplified by PCR using 4 μl of 5X Green GoTaq Flexi Buffer, 0.4 μl of 10 mM dNTPs, 0.1 μl of 500 units TaqDNA polymerase, 1.2 μl of 25 mM MgCl_2 (Promega), and 0.4 μl of each 20 pM of specific sense and antisense primer of COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR products were analyzed on 1.2% agarose gel. The primer sequences used by PCR were as follows: COX-2, forward, 5'-CTG TAC TAC GCC GAG ATT CCT GA-3' and reverse, 5'-GTC CTC GCT TCT GAT CTG TCT TG-3'; GAPDH, forward, 5'-GGT GAA GGT CGG TGT GAA CG-3' and reverse, 5'-GGT AGG AAC ACG GAA GGC CA-3'. The PCR conditions applied were: COX-2, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec; GAPDH, 27 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec, respectively. GAPDH was used as an internal control to evaluate the relative expression of COX-2.

Measurement of COX-2 protein stability. C6 cells were then treated with cadmium alone or cadmium plus MG132 for

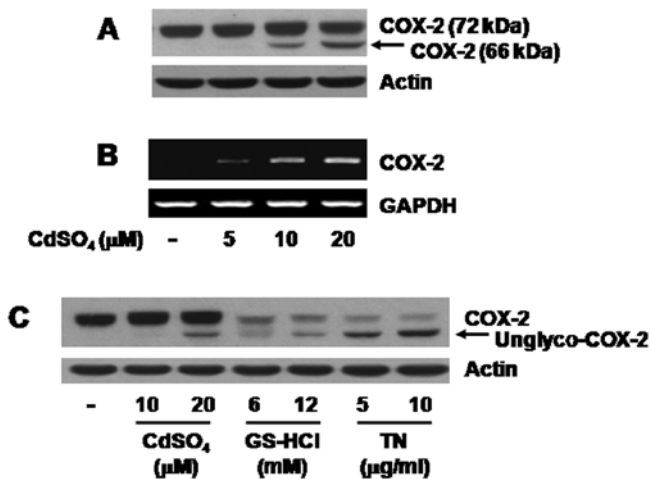


Figure 1. Effect of cadmium on expression of COX-2 protein and mRNA in C6 cells. (A and B) C6 cells were treated without or with the indicated concentrations of cadmium for 8 h. Whole cell lysates and total-RNA were prepared and subjected to (A) western blot analysis and (B) RT-PCR, respectively. (C) C6 cells were treated without or with the indicated doses of cadmium or known inhibitor of protein N-glycosylation, such as glucosamine-hydrochloride (GS-HCl) or tunicamycin (TN), for 8 h. Whole cell lysates were prepared and subjected to western blot analysis. Data are representative of three independent experiments.

2, 4 or 8 h in the absence or presence of CHX, a translation inhibitor, to block ongoing translation. 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 remaining in the cells.

Results

Cadmium treatment increases not only expression of COX-2 mRNA but also expression of both N-glycosylated and unglycosylated COX-2 protein in C6 cells. Initially, the effect of different concentrations of cadmium on expression of COX-2 protein in C6 cells was analyzed by western blotting. As shown in Fig. 1A (upper panel), treatment with cadmium at 5 μ M for 8 h did not induce expression of COX-2 protein while that with cadmium at 10 or 20 μ M increased expression of COX-2 proteins of 72 and 66 kDa molecular mass. RT-PCR experiments were next carried out to evaluate the effect of cadmium on expression of COX-2 mRNA. As shown in Fig. 1B (upper panel), treatment with cadmium for 8 h increased expression of COX-2 mRNA in a concentration-dependent manner. We next tested the effect of cadmium on N-glycosylation of COX-2 in C6 cells. To accomplish this, TN or GS-HCl that inhibits COX-2 N-glycosylation (23,26), was used as the positive control. As shown in Fig. 1C (upper panel), the size (66 kDa) of COX-2 induced by cadmium was nearly identical to that of COX-2 induced by GS-HCl or TN in the C6 cells. Expression of control actin protein or GAPDH mRNA remained unchanged under these experimental conditions (Fig. 1A-C, lower panels). Due to the strongest effect on expression of COX-2 proteins (72 and 66 kDa) and COX-2 mRNA, we selected 20 μ M of cadmium for further study.

Kinetics of the expression and/or activation of COX-2 and ATF-2 in the cadmium-treated C6 cells. Kinetic studies were

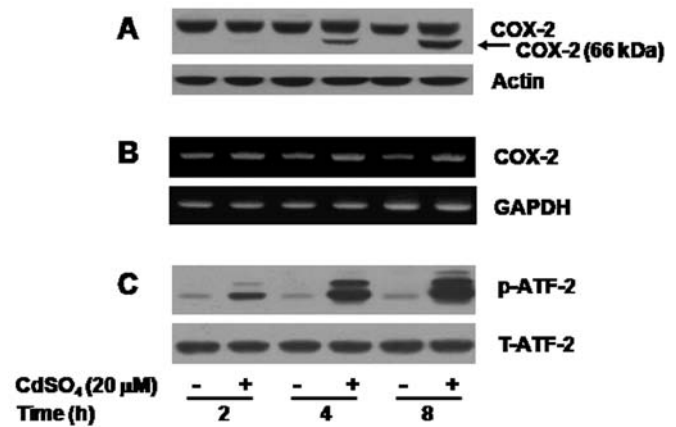


Figure 2. Time course experiments on expression of COX-2 protein and mRNA, and activation of ATF-2 in the cadmium-treated C6 cells. (A-C) C6 cells were treated without or with cadmium (20 μ M) for the indicated times. Whole cell lysates and total-RNA were prepared and subjected (A and C) to western blot analysis and (B) RT-PCR, respectively. Data are representative of three independent experiments. p-ATF-2, phosphorylated ATF-2; T-ATF-2, total ATF-2.

next performed to determine the time of induction of COX-2 proteins and mRNA by cadmium. Cadmium treatment at 2 h did not modulate expression of COX-2 protein while there was a time-dependent induction of both N-glycosylated and unglycosylated COX-2 proteins at the cadmium treatment of 4 or 8 h (Fig. 2A, upper panel). In case of COX-2 mRNA, treatment with cadmium at 2 h slightly increased expression of COX-2 mRNA (Fig. 2B, upper panel). There was a further enhancement of COX-2 mRNA expression at 4 or 8 h. Expression of actin protein or GAPDH mRNA remained unchanged during these experimental conditions (Fig. 2A and B, lower panels). The effect of cadmium on activation of TFs, herein ATF-2, in C6 cells was next investigated. Cadmium treatment led to a strong time-dependent activation of ATF-2 in C6 cells (Fig. 2C, upper panel). Expression of total ATF-2 protein remained unchanged during these experimental conditions (Fig. 2C, lower panel), suggesting that cadmium treatment increases phosphorylation of the preexisting ATF-2 protein without *de novo* protein synthesis.

Stability of both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium in C6 cells is 26S proteasome-dependent and the cadmium-induced expression of both forms of COX-2 occurs in a translation-dependent manner. We next determined the stability of both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium in C6 cells using protein stability assay with CHX, a protein synthesis inhibitor. When translation was blocked in the presence of CHX, the amount of both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium was sharply decreased in a time-dependent manner, suggesting that when translation is inhibited, both forms of COX-2 induced by cadmium are rapidly degraded (Fig. 3A, upper panel). Importantly, the degradation was effectively blocked by treatment with MG132, a 26S proteasomal inhibitor. However, when translation was ongoing (no CHX), there was expression of both N-glycosylated and unglycosylated COX-2 proteins induced

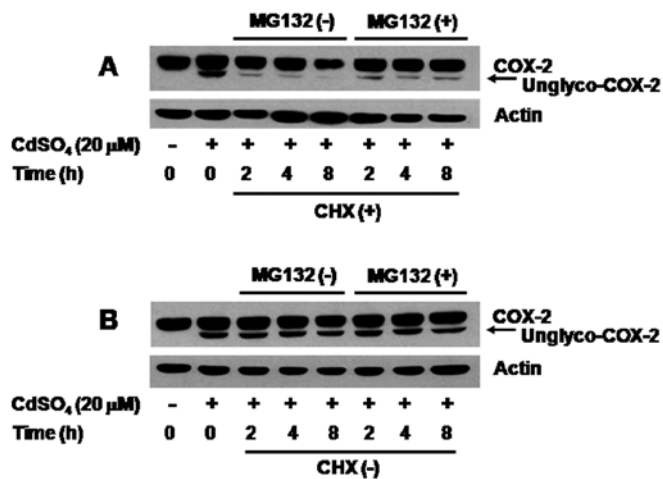


Figure 3. Effect of CHX or MG132 on the stability and/or expression of both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium in C6 cells. C6 cells were initially treated without or with cadmium (20 μM) for 8 h to highly induce COX-2 protein and were then exposed to cadmium without or with MG132 (4 μM), a 26S proteasomal inhibitor, in the (B) absence or (A) presence of cycloheximide (CHX), a translational inhibitor, for the indicated times. At each time, whole cell lysates were prepared and analyzed by western blot analysis using antibodies for COX-2 or actin to measure the amounts of COX-2 protein remained in the cells at the respective time. Data are representative of three independent experiments.

by cadmium in C6 cells that were grown in the absence or presence of MG132 (Fig. 3B, upper panel). Expression of actin protein remained unchanged under these experimental conditions (Fig. 3, lower panels).

Cadmium-induced COX-2 transcriptional upregulation in C6 cells is closely related to oxidative stress and activation of the family of MAPKs. Previously, studies have shown the cadmium-mediated expression of COX-2 through oxidative stress (27,28). This promptly led us to investigate the role of oxidative stress in the cadmium-induced COX-2 expression in C6 cells using sulfhydryl group-containing reducing agents (NAC, GSH and an antioxidant VE). Pretreatment with NAC or GSH, but not with VE, strongly suppressed the cadmium-induced expression of both N-glycosylated and unglycosylated COX-2 proteins (Fig. 4A, upper panel). We further determined whether intracellular signaling proteins participate in the cadmium-induced COX-2 expression in C6 cells by use of the specific pharmacological inhibitor of ERK-1/2 (PD98059, PD), JNK-1/2 (SP600125, SP), p38 MAPK (SB203580, SB) or PI3K (LY294002, LY). Pretreatment with PD98059, SP600125 or SB203580 effectively blocked the cadmium-induced expression of both forms of COX-2 while that with LY294002 had little effect (Fig. 4C, upper panel). Results of RT-PCR analyses, demonstrated that pretreatment with NAC, GSH, PD98059, SP600125 or SB203580 suppressed the cadmium-induced COX-2 mRNA expression (Fig. 4B and D, upper panels). Expression of actin protein or GAPDH mRNA remained unchanged under these experimental conditions (Fig. 4, lower panels).

Oxidative stress lies upstream of activation of the family of MAPKs in the cadmium-treated C6 cells. We next investigated any link between oxidative stress and activation of the

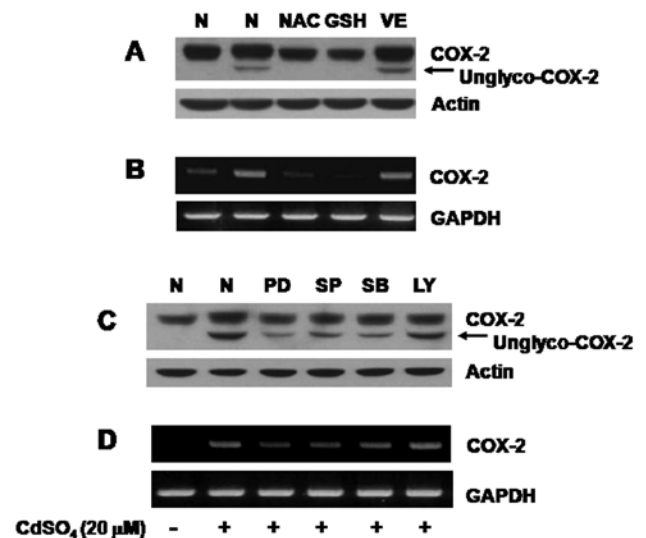


Figure 4. Effect of NAC, GSH or VE on expression of COX-2 protein and mRNA in the cadmium-treated C6 cells. (A and B) C6 cells were pretreated without or with N-acetyl-cysteine (NAC, 10 mM), glutathione (GSH, 10 mM) or vitamin E (VE, 100 μM) for 1 h and then treated without or with cadmium (20 μM) in the absence or presence of each agent for an additional 8 h. Whole cell lysates were prepared and analyzed by (A) western blot analysis and (B) RT-PCR, respectively. (C and D) C6 cells were pretreated without or with PD98059 (PD, 50 μM), SP600125 (SP, 25 μM), SB203580 (SB, 25 μM) or LY294002 (LY, 25 μM) for 1 h and then treated without or with cadmium (20 μM) in the absence or presence of each inhibitor for an additional 8 h. Whole cell lysates were prepared and analyzed by (C) western blot analysis and (D) RT-PCR, respectively. Data are representative of three independent experiments.

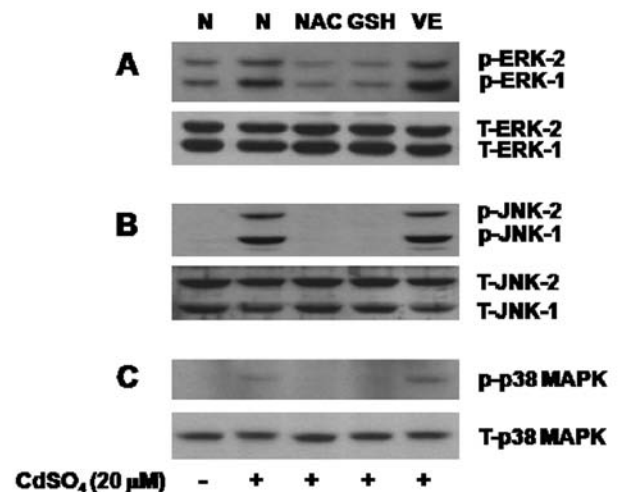


Figure 5. Effect of NAC, GSH, or VE on activation of ERK-1/2, JNK-1/2, and p38 MAPK in the cadmium-treated C6 cells. (A-C) C6 cells were pretreated without or with N-acetyl-cysteine (NAC, 10 mM), glutathione (GSH, 10 mM) or vitamin E (VE, 100 μM) for 1 h and then treated without or with cadmium (20 μM) in the absence or presence of each agent for an additional 8 h. Whole cell lysates were prepared and analyzed by western blot analysis. Data are representative of three independent experiments. p-ERK-1/2, phosphorylated ERK-1/2; T-ERK-1/2, total ERK-1/2; p-JNK-1/2, phosphorylated JNK-1/2; T-JNK-1/2, total JNK-1/2; p-p38 MAPK, phosphorylated p38 MAPK; T-p38 MAPK, total p38 MAPK.

family of MAPKs in the cadmium-treated C6 cells. Cadmium treatment for 4 h increased phosphorylation of ERK-1/2, p38 MAPK and JNK-1/2 in C6 cells (Fig. 5, upper panels).

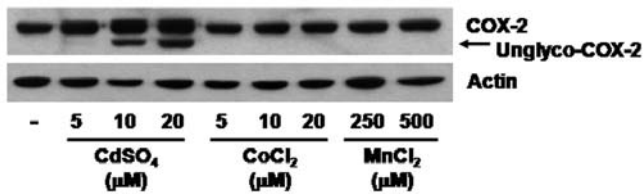


Figure 6. Comparison of the effect of cadmium or other heavy metals on expression of COX-2 in C6 cells. (A) C6 cells were treated without or with the indicated concentrations of cadmium or other heavy metals, such as cobalt-chloride or manganese-chloride, for 8 h. Whole cell lysates were prepared and subjected to western blot analysis. Data are representative of three independent experiments.

However, cadmium treatment did not affect total expression of each member of the MAPKs in C6 cells, suggesting that cadmium treatment also enhances phosphorylation of the preexisting MAPKs in C6 cells without *de novo* protein synthesis (Fig. 5, lower panels). Interestingly, pretreatment with NAC or GSH, but not VE, strongly interfered with the cadmium-mediated phosphorylation of ERK-1/2, p38 MAPK and JNK-1/2 in C6 cells.

Cadmium specifically induces expression of both N-glycosylated and unglycosylated forms of COX-2 in C6 cells. To evaluate the specificity, C6 cells were treated with cadmium or different heavy metals (cobalt or manganese) and expression of COX-2 in the conditioned cells was measured. Treatment with cadmium (10 or 20 μ M) induced expression of both N-glycosylated and unglycosylated COX-2 proteins, while there was no induction of COX-2 by treatment with cobalt (5, 10 or 20 μ M) or manganese (250 or 500 μ M) in C6 cells (Fig. 6, upper panel). Expression of actin protein remained unchanged under these experimental conditions (Fig. 6, lower panel).

Discussion

Cadmium is a toxic heavy metal and an environmental hazard to humans. Deregulated expression of COX-2 is linked to many neuronal diseases, including inflammation and cancer (29,30). In this study, we investigated the effect of cadmium on expression of COX-2 in C6 rat glioma cells.

Previously, the cadmium-mediated upregulation of COX-2 protein expression was shown (27,31). COX-2 is an N-glycoprotein and has four glycosylation sites (asparagine) within the structure (21,22). In general, protein N-glycosylation occurs during translation and is important for protein stability, activity, and/or cellular localization (32). Based on this fact, it was previously shown that exposure to GS-HCl or TN, inhibitors of protein N-glycosylation, prevents COX-2 N-glycosylation leading to generation of the unglycosylated COX-2 with decreased molecular mass and enzymatic activity (23,26). In this study, cadmium induced expression not only of the normally expressed COX-2 of 72 kDa (N-glycosylated COX-2) but also the unglycosylated COX-2 of 66 kDa in C6 cells (Fig. 1A), as assessed by the unglycosylated COX-2 induced by GS-HCl or TN (Fig. 1C). However, considering the magnitude on expression of the unglycosylated COX-2

induced by cadmium and the known inhibitor of COX-2 N-glycosylation, it seems that cadmium only partially interferes with N-glycosylation of COX-2 in C6 cells. Moreover, taking into account a previous study that cadmium perturbs COX-2 N-glycosylation in H4 neuronal cells (27), it is unlikely that the cadmium-mediated expression of the unglycosylated COX-2 is limited to C6 cells. The regulation of COX-2 turnover is an important contributor to COX-2 expression. The $t_{1/2}$ of COX-2 protein in cells is suggested to be within hours. There is evidence suggesting a 26S proteasome-dependent COX-2 protein turnover (33). Notably, data of CHX-based protein stability assays herein demonstrated that both N-glycosylated and unglycosylated COX-2 proteins induced by cadmium are rapidly degraded while their degradation is effectively blocked by MG132 in the presence of CHX (Fig. 3A), which suggests that both forms of COX-2 induced by cadmium are labile and the proteasomal pathway is involved in their turnover. In addition, the present study further demonstrated that cadmium can induce and maintain expression of the unglycosylated COX-2 protein in C6 cells in the absence of CHX (Fig. 3B), implying that cadmium induces expression of the unglycosylated COX-2 protein by targeting N-glycosylation of nascent COX-2 polypeptide. To the best of our knowledge, this is the first report of the proteasome- and translation-dependent regulation of the expression of the N-glycosylated and unglycosylated COX-2 proteins by cadmium.

Our data also indicate cadmium-induced COX-2 transcriptional upregulation in C6 cells (Figs. 1B and 2B). COX-2 transcriptional induction was found to be largely affected by activities of TFs (7,10,16,17) and signaling proteins (34,35). Previous results from our laboratory demonstrated that cadmium does not induce activation of NF- κ B but triggers activation of ERK-1/2, p38 MAPK, and JNK-1/2 in C6 cells (36). In this study, we demonstrated for the first time that cadmium has the ability to rapidly induce activation of ATF-2 in C6 cells (Fig. 2C). In a recent study, the activation of p38 MAPK, but not JNK-1/2, was crucial for the cadmium-induced COX-2 expression in H4 cells (27). However, the present study showed that cadmium-induced COX-2 expression in C6 cells was dependent on the activation not only of p38 MAPK but also ERK-1/2 and JNK-1/2 (Fig. 4C and D). There is evidence suggesting that p38 MAPK and/or JNK-1/2 regulate activation of ATF-2 (37,38) and ERK-1/2 and/or JNK-1/2 are involved in AP-1 activation (39,40). A recent finding revealed the effect of cadmium on the rapid upregulation of c-fos and c-jun, two key components of the AP-1 transcription factor, in HPT human proximal tubule cells of the kidney (41). Although speculative, it is thus likely that the cadmium-induced COX-2 transcriptional upregulation in C6 cells is in part mediated through the p38 MAPK/JNK-1/2-ATF-2 and ERK-1/2/JNK-1/2-AP-1 pathways.

Early studies indicated a link between oxidative stress and COX-2 expression (42,43). Oxidative stress often occurs due to an imbalance in intracellular levels of oxidants (e.g., ROS) and reducing agents (e.g., GSH). Previous studies demonstrated a cadmium-mediated oxidative stress by decreasing cellular GSH and/or increasing cellular ROS (44,45). Supporting these findings, we previously demonstrated that cadmium rapidly lowers cellular GSH and the GSH reduction is important for the metal-mediated expression of MAPK phosphatase-1 in C6

cells (36). In this study, we further showed that oxidative stress probably linked to a reduction in cellular GSH is critical for COX-2 transcriptional upregulation by cadmium in C6 cells (Fig. 4A and B). It is known that oxidative stress lies upstream of the activation of TFs and/or signaling proteins in cadmium-induced COX-2 expression and apoptotic death of neuronal cells (28). The present study also suggests crosstalk between oxidative stress and the activation of the family of MAPKs in cadmium-treated C6 cells (Fig. 5), in which cadmium treatment primarily lowers cellular GSH, which subsequently leads to activation of p38 MAPK, ERK-1/2 and JNK-1/2.

The specificity of cadmium to induce expression of both N-glycosylated and unglycosylated forms of COX-2 in C6 cells was also noted. This notion is based on the absence of induction of COX-2 protein in C6 cells exposed to other heavy metals, such as cobalt or manganese (Fig. 6). However, previous reports have demonstrated cobalt- or manganese-mediated upregulation of COX-2 expression in various cell types, in which expression of COX-2 protein was increased in vector or phospholipase D cDNA-transfected U87 MG human astrogloma cells treated with cobalt (200 μ M, 20 h) (46) or in PC3 human prostate cancer cells exposed to cobalt (100 μ M, 12 h) (47). Strong COX-2 protein expression was also observed in cultured astrocytes treated with manganese (100 μ M, 16 h) (48) and in A549 human airway cells exposed to manganese (100 μ M, 16 h) (25). It is plausible that the differential effect on COX-2 expression by cobalt or manganese in the present study and previous reports may be due to the use of differential experimental systems, including cDNA transfection, treatment concentration and time of each metal and/or different type of cells used.

It is now obvious that cadmium induces expression of both N-glycosylated and unglycosylated COX-2 in C6 cells. However, the biological role (or relevance) of the cadmium-mediated expression of both forms of COX-2 in C6 cells remains unclear at this moment. However, assuming that COX-2 expression (and activity) contributes to the growth of glioma (14) and is linked to neuroinflammation (30), it is conceivable that the N-glycosylated COX-2 induced by cadmium may contribute to C6 cell growth and/or induce a pro-inflammatory response. It is well documented that inhibition of protein N-glycosylation leads to production of misfolded and/or nonfunctional proteins (32) and cellular accumulation of such damaged proteins elicits ER stress (49,50). Thus, it is suggested that accumulation of the unglycosylated proteins (herein COX-2) induced by cadmium may contribute to ER stress in C6 cells.

In conclusion, we demonstrate for the first time that cadmium induces expression of COX-2 through both transcriptional and co-translational (N-glycosylation) regulation in C6 cells in which the cadmium-induced COX-2 transcriptional upregulation is closely related to oxidative stress-dependent activation of the family of MAPKs and the cadmium-induced expression of both N-glycosylated and unglycosylated COX-2 proteins occurs in the proteasome- and/or translation-dependent control.

Acknowledgements

The present study was supported by the Bisa Research Grant of Keimyung University in 2010.

References

- Gerhardsson L, Englyst V, Lundström NG, *et al*: Cadmium, copper and zinc in tissues of deceased copper smelter workers. *J Trace Elem Med Biol* 16: 261-266, 2002.
- Satarug S, Garrett SH, Sens MA and Sens DA: Cadmium, environmental exposure, and health outcomes. *Environ Health Perspect* 118: 182-190, 2010.
- Bertin G and Averbeck D: Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (Review). *Biochimie* 88: 1549-1559, 2006.
- Im JY, Paik SG and Han PL: Cadmium-induced astroglial death proceeds via glutathione depletion. *J Neurosci Res* 83: 301-308, 2006.
- Liu J, Qu W and Kadiiska MB: Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicol Appl Pharmacol* 238: 209-214, 2009.
- Matés JM, Segura JA, Alonso FJ and Márquez J: Roles of dioxins and heavy metals in cancer and neurological diseases using ROS-mediated mechanisms. *Free Radic Biol Med* 49: 1328-1341, 2010.
- Smith WL, DeWitt DL and Garavito RM: Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 69: 145-182, 2000.
- Smith WL and Dewitt DL: Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 62: 167-215, 1996.
- Vane JR, Bakhle YS and Botting RM: Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 38: 97-120, 1998.
- Smith WL, Garavito RM and DeWitt DL: Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 271: 33157-33160, 1996.
- Williams GW: An update on nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors. *Curr Pain Headache Rep* 9: 377-389, 2005.
- Strauss KI, Barbe MF, Marshall RM, *et al*: Prolonged cyclooxygenase-2 induction in neurons and glia following traumatic brain injury in the rat. *J Neurotrauma* 17: 695-711, 2000.
- Maihöfner C, Probst-Cousin S, Bergmann M, *et al*: Expression and localization of cyclooxygenase-1 and -2 in human sporadic amyotrophic lateral sclerosis. *Eur J Neurosci* 18: 1527-1534, 2003.
- Joki T, Heese O, Nikas DC, *et al*: Expression of cyclooxygenase 2 (COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398. *Cancer Res* 60: 4926-4931, 2000.
- Herschman HR, Reddy ST and Xie W: Function and regulation of prostaglandin synthase-2. *Adv Exp Med Biol* 407: 61-66, 1997.
- Newton R, Kuitert LM, Bergmann M, *et al*: Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem Biophys Res Commun* 237: 28-32, 1997.
- Inoue H, Yokoyama C, Hara S, *et al*: Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* 270: 24965-24971, 1995.
- Ristimäki A, Garfinkel S, Wessendorf J, *et al*: Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *J Biol Chem* 269: 11769-11775, 1994.
- Srivastava SK, Tetsuka T, Daphna-Iken D and Morrison AR: IL-1 beta stabilizes COX II mRNA in renal mesangial cells: role of 3'-untranslated region. *Am J Physiol* 267: F504-F508, 1994.
- Jang BC, Muñoz-Najar U, Paik JH, *et al*: Leptomycin B, an inhibitor of the nuclear export receptor CRM1, inhibits COX-2 expression. *J Biol Chem* 278: 2773-2776, 2003.
- Nemeth JF, Hochgesang GP Jr, Marnett LJ and Caprioli RM: Characterization of the glycosylation sites in cyclooxygenase-2 using mass spectrometry. *Biochemistry* 40: 3109-3116, 2001.
- Hla T and Neilson K: Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* 89: 7384-7388, 1992.
- Otto JC, DeWitt DL and Smith WL: N-glycosylation of prostaglandin endoperoxide synthases-1 and -2 and their orientations in the endoplasmic reticulum. *J Biol Chem* 268: 18234-18242, 1993.
- Tung WH, Hsieh HL and Yang CM: Enterovirus 71 induces COX-2 expression via MAPKs, NF-kappaB, and AP-1 in SK-N-SH cells: Role of PGE(2) in viral replication. *Cell Signal* 22: 234-246, 2010.
- Jang BC: Induction of COX-2 in human airway cells by manganese: role of PI3K/PKB, p38 MAPK, PKCs, Src, and glutathione depletion. *Toxicol In Vitro* 23: 120-126, 2009.

26. Jang BC, Sung SH, Park JG, *et al*: Glucosamine hydrochloride specifically inhibits COX-2 by preventing COX-2 N-glycosylation and by increasing COX-2 protein turnover in a proteasome-dependent manner. *J Biol Chem* 282: 27622-27632, 2007.
27. Figueiredo-Pereira ME, Li Z, Jansen M and Rockwell P: N-acetylcysteine and celecoxib lessen cadmium cytotoxicity which is associated with cyclooxygenase-2 upregulation in mouse neuronal cells. *J Biol Chem* 277: 25283-25289, 2002.
28. Rockwell P, Martinez J, Papa L and Gomes E: Redox regulates COX-2 upregulation and cell death in the neuronal response to cadmium. *Cell Signal* 16: 343-353, 2004.
29. Hara A and Okayasu I: Cyclooxygenase-2 and inducible nitric oxide synthase expression in human astrocytic gliomas: correlation with angiogenesis and prognostic significance. *Acta Neuropathol* 108: 43-48, 2004.
30. Choi SH, Aid S and Bosetti F: The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research. *Trends Pharmacol Sci* 30: 174-181, 2009.
31. Park CS, Kim OS, Yun SM, *et al*: Presenilin 1/gamma-secretase is associated with cadmium-induced E-cadherin cleavage and COX-2 gene expression in T47D breast cancer cells. *Toxicol Sci* 106: 413-422, 2008.
32. Roth J, Zuber C, Park S, *et al*: Protein N-glycosylation, protein folding, and protein quality control. *Mol Cells* 30: 497-506, 2010.
33. Singh M, Chaudhry P, Parent S and Asselin E: Ubiquitin-proteasomal degradation of COX-2 in TGF- β stimulated human endometrial cells is mediated through endoplasmic reticulum mannosidase I. *Endocrinology* 153: 426-437, 2012.
34. Pham H, Vincenti R and Slice LW: COX-2 promoter activation by AT1R-Gq-PAK-p38 β signaling in intestinal epithelial cells. *Biochim Biophys Acta* 1779: 408-413, 2008.
35. Healy ZR, Zhu F, Stull JD and Konstantopoulos K: Elucidation of the signaling network of COX-2 induction in sheared chondrocytes: COX-2 is induced via a Rac/MEKK1/MKK7/JNK2/c-Jun-C/EBP β -dependent pathway. *Am J Physiol Cell Physiol* 294: C1146-C1157, 2008.
36. Kim SM, Park JG, Baek WK, *et al*: Cadmium specifically induces MKP-1 expression via the glutathione depletion-mediated p38 MAPK activation in C6 glioma cells. *Neurosci Lett* 440: 289-293, 2008.
37. Chen KC, Chiou YL and Chang LS: JNK1/c-Jun and p38 α MAPK/ATF-2 pathways are responsible for upregulation of Fas/FasL in human chronic myeloid leukemia K562 cells upon exposure to Taiwan cobra phospholipase A2. *J Cell Biochem* 108: 612-620, 2009.
38. Tindberg N, Porsmyr-Palmertz M and Simi A: Contribution of MAP kinase pathways to the activation of ATF-2 in human neuroblastoma cells. *Neurochem Res* 25: 527-531, 2000.
39. Ho BY, Wu YM, Chang KJ and Pan TM: Dimeric acid inhibits SW620 cell invasion by attenuating H₂O₂-mediated MMP-7 expression via JNK/C-Jun and ERK/C-Fos activation in an AP-1-dependent manner. *Int J Biol Sci* 7: 869-880, 2011.
40. Ding J, Huang Y, Ning B, *et al*: TNF- α induction by nickel compounds is specific through ERKs/AP-1-dependent pathway in human bronchial epithelial cells. *Curr Cancer Drug Targets* 9: 81-90, 2009.
41. Garrett SH, Phillips V, Somji S, *et al*: Transient induction of metallothionein isoform 3 (MT-3), c-fos, c-jun and c-myc in human proximal tubule cells exposed to cadmium. *Toxicol Lett* 126: 69-80, 2002.
42. Yang C, Ling H, Zhang M, *et al*: Oxidative stress mediates chemical hypoxia-induced injury and inflammation by activating NF- κ B-COX-2 pathway in HaCaT cells. *Mol Cells* 31: 531-538, 2011.
43. Yoon JH, Lim TG, Lee KM, *et al*: Tangeretin reduces ultraviolet B (UVB)-induced cyclooxygenase-2 expression in mouse epidermal cells by blocking mitogen-activated protein kinase (MAPK) activation and reactive oxygen species (ROS) generation. *J Agric Food Chem* 59: 222-228, 2011.
44. Yang MS, Li D, Lin T, *et al*: Increase in intracellular free/bound NAD(P)H as a cause of Cd-induced oxidative stress in the HepG(2) cells. *Toxicology* 247: 6-10, 2008.
45. Pathak N and Khandelwal S: Influence of cadmium on murine thymocytes: potentiation of apoptosis and oxidative stress. *Toxicol Lett* 165: 121-132, 2006.
46. Ahn BH, Park MH, Lee YH, *et al*: Upregulation of cyclooxygenase-2 by cobalt chloride-induced hypoxia is mediated by phospholipase D isozymes in human astrogloma cells. *Biochim Biophys Acta* 1773: 1721-1731, 2007.
47. Liu XH, Kirschenbaum A, Yao S, *et al*: Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clin Exp Metastasis* 17: 687-694, 1999.
48. Liao SL, Ou YC, Chen SY, *et al*: Induction of cyclooxygenase-2 expression by manganese in cultured astrocytes. *Neurochem Int* 50: 905-915, 2007.
49. Hong M, Luo S, Baumeister P, *et al*: Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response. *J Biol Chem* 279: 11354-11363, 2004.
50. Yu SM and Kim SJ: Endoplasmic reticulum stress (ER-stress) by 2-deoxy-D-glucose (2DG) reduces cyclooxygenase-2 (COX-2) expression and N-glycosylation and induces a loss of COX-2 activity via a Src kinase-dependent pathway in rabbit articular chondrocytes. *Exp Mol Med* 42: 777-786, 2010.