

# Transcriptional Regulation of Cyclooxygenase-1 by Histone Deacetylase Inhibitors in Normal Human Astrocyte Cells\*

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While cyclooxygenase (COX)-2 is a highly inducible gene, COX-1 is widely known as a noninducible gene and is constitutively expressed in a variety of cell lines and human tissues. Recently, several reports have indicated that COX-1 is also regulated at the transcriptional level by various stimuli. We present evidence that histone deacetylase (HDAC) inhibitors induce COX-1 transcription and translation in normal human astrocyte (NHA) cells and glioma cell lines. HDAC inhibitors increased acetylated histone H4 protein expression in NHA cells. The levels of COX-1 mRNA and protein were maximal at 24 and 48 h, respectively, after treatment with the specific HDAC inhibitor, trichostatin A (TSA). In addition, TSA-treated NHA cells produced prostaglandin E<sub>2</sub> as determined by enzyme-linked immunosorbent assay after incubation with 10 μM exogenous arachidonic acid, indicating that the induced COX-1 is functionally active. In addition to NHA cells, this up-regulation of COX-1 after treatment with HDAC inhibitors was observed in 5 different glioma cell lines. The nucleotide sequence of the inducible COX-1 cDNA was confirmed identical to human COX-1 that was previously reported. HDAC inhibitors stimulated COX-1 promoter activity as measured by luciferase reporter assays, suggesting that the induction of COX-1 is regulated at the transcriptional level. Furthermore, mutation analysis of the COX-1 promoter suggests that TSA-responsive element exists in the proximal Sp1-binding site at +25 to +31. In conclusion, COX-1 is an inducible gene in glial-derived cells including immortalized cells, and appears to be transcriptionally regulated by a unique mechanism associated with histone acetylation.

stimuli. Expression of COX-2 can be induced by cytokines, growth factors, tumor promoters, and bacterial endotoxins (1–4).

Histones are core proteins of nucleosomes and acetylation of nuclear histones is regulated by histone acetyltransferase and histone deacetylase (HDAC) (5–7). Binding of transcriptional factors to DNA recruits histone acetyltransferase proteins that lead to the acetylation of core histone, enhance nucleosomal relaxation, and subsequently induce transcription (8). On the other hand, several transcriptional factors, CBF (9), hormone-dependent nuclear receptors (10), and Mad (11), can bind to HDAC, which stabilizes nucleosomal structure and repress transcription (12). Thus, histone acetylation is known to be associated with transcriptional activity in eukaryotic cells. For example, HDAC inhibitors consistently induce the cyclin-dependent kinase inhibitor p21<sup>WAF1/Cip1</sup> (13–16), which causes cell growth arrest in various tumor cell lines. In general, inhibition of HDAC results in accumulation of acetylated histone protein (15, 17, 18). Furthermore, inhibition of HDAC is known to affect a variety of biological processes, such as the induction of differentiation (19, 20), cell cycle arrest (13, 16–18, 21), and apoptotic cell death (19, 22). HDAC inhibitors, which cause the induction of apoptosis or differentiation, serve as the basis for the development new drugs with potential for the treatment of cancer (15, 17, 20, 21).

Previously, our laboratory demonstrated that an increase in histone acetylation by HDAC inhibitors decreases COX-2 expression and increases 15-lipoxygenase-1 (15-LO-1) expression, apoptosis, and differentiation in human colorectal carcinoma cells (19, 23). The expression of 15-LO-1 in human colorectal epithelial cells, the high expression of 15-LO-1 in tumors, and the link between 15-LO-1 expression and histone acetylation was found. Moreover, the acetylation of histone H3 and STAT6 is required for transcriptional activation of the 15-LO-1 gene (24).

In this report we have investigated the effects of HDAC inhibitors on the expression of lipid metabolizing enzymes, COX-1, COX-2, and 15-LO-1, in human brain cell lines. In the brain, COX-2 plays an important role in various pathological conditions (25, 26) such as ischemia, seizure, and injury, and has been proposed to contribute to the development of Alzheimer's disease (27, 28). On the other hand, the expression of COX-1 is regulated during brain development (29), and COX-1 is also up-regulated by retinoic acid during neuronal differentiation in neuroblastoma cell lines (30). However, little else is known about the regulation of COX-1 expression in the human brain. We selected the normal human astrocyte (NHA) cell line for this study for several reasons. 1) Astrocytes are the major cell population in the central nervous system (31), and, reports using human astrocytes are lacking. 2) Activated astrocytes produce cytokines, and these factors eventually lead to neuro-

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Cyclooxygenase (COX)<sup>1</sup> is the key enzyme in the metabolic pathway leading to prostaglandins (PGs) and thromboxane A<sub>2</sub> formation from arachidonic acid. COX exists as two isoforms: COX-1 is constitutively expressed as a housekeeping gene, while COX-2 is a highly inducible gene in response to various

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<sup>1</sup> The abbreviations used are: COX, cyclooxygenase; 15-LO-1, 15-lipoxygenase-1; EIA, enzyme immunoassay; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; NaBT, sodium butyrate; NHA, normal human astrocyte; PG, prostaglandin; PBS, phosphate-buffered saline; TSA, trichostatin A; FBS, fetal bovine serum; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

nal damage (25, 27). 3) Last, prostaglandins and lipid metabolites formed by astrocytes may contribute to central nervous system pathology and physiology (26, 31, 32). Here, we report for the first time that HDAC inhibitors induce only COX-1 expression, not COX-2, in normal human astrocyte cells and glioma cell lines.

#### EXPERIMENTAL PROCEDURES

**Materials**—The NHA cell line was obtained from Clonetics (San Diego, CA). The human glioma cell lines (A172, T98G, U87MG, U138MG, and U373MG) and the human colorectal carcinoma cell line Caco-2 were obtained from the American Type Culture Collection (Rockville, MD). Interleukin- $\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  were obtained from R&D system (Minneapolis, MN). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT) and sodium butyrate (NaBT), trichostatin A (TSA), HC toxin, 12-*O*-tetradecanoylphorbol-13-acetate, benzo(a)pyrene (B(a)P), benzo(a)pyrene diol-epoxide, and all other chemicals were obtained from Sigma.

**Cell Culture**—NHA cells were grown in astrocyte growth medium from Clonetics and cells from passages 3 to 7 were used in these experiments. The glioma cell lines and Caco-2 cells were grown in Eagle's minimal essential medium containing 10% FBS, 1 mM sodium pyruvate (Invitrogen, Rockville, MD), and gentamicin (10  $\mu$ g/ml), and cells from passages 5 to 15 were used. In these studies, TSA and HC toxin were dissolved in Me<sub>2</sub>SO, and NaBT was dissolved in phosphate-buffered saline (PBS).

**Immunoblot Analysis**—The semiconfluent cells on 100-mm diameter dishes were washed twice with ice-cold PBS and lysed in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were then sonicated on ice four times for 20 s at 50% power. Protein content was quantified by the bicinchoninic acid method using BCA protein assay reagent (Pierce, Rockford, IL). Aliquots of the 20  $\mu$ g of protein were boiled in protein sample buffer (9% SDS, 15% glycerol, 30 mM Tris-HCl, pH 7.8, 0.05% bromophenol blue, 6%  $\beta$ -mercaptoethanol) and separated on SDS-PAGE using 8% acrylamide gels. Ovine COX-1 purified protein (Cayman, Ann Arbor, MI) and murine recombinant COX-2 protein (Cayman) were also loaded on the gel with the experimental samples for positive control. After electrophoretic transfer of the protein from the polyacrylamide gel to nitrocellulose membrane, the membrane was blocked by incubating with 10% dry milk (Bio-Rad) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) overnight at 4 °C. After being washed three times in TBS-T, the membrane was probed with monoclonal anti-human COX-1 mouse antibody (Cayman) diluted 1:4000 in TBS-T, 1% dry milk, polyclonal anti-human COX-2 rabbit antibody (Oxford Biomedical Research, Oxford, MI) diluted 1:4000 in TBS-T, 1% dry milk, or with polyclonal anti-human goat actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000 in TBS-T, 1% dry milk for 1 h at room temperature. The membrane was then washed three times with TBS-T and incubated for 1 h at room temperature with 1:5000 dilution of peroxidase-conjugated anti-mouse (Amersham Biosciences, Arlington Heights, IL), anti-rabbit (Amersham Biosciences), or anti-goat (Santa Cruz) immunoglobulin antibody in TBS-T, 1% dry milk. The membrane was washed three more times with TBS-T and immunocomplex was visualized by enhanced chemiluminescence using the ECL kit (Amersham Biosciences).

**Histone Isolation**—The semiconfluent cells on 150-mm diameter dishes were washed twice with ice-cold PBS and lysed with 1 ml of lysis buffer (10 mM Tris-HCl (pH 6.5), 50 mM sodium bisulfite, 10 mM MgCl<sub>2</sub>, 10 mM sodium butyrate, 8.6% sucrose, 1% Triton X-100) and homogenized by Dounce homogenizer (KONETES GLASS CO., Vineland, NJ). The homogenates were centrifuged at 1,000  $\times$  g for 5 min at 4 °C, and the pellets were washed with 0.5 ml of suspension buffer (10 mM Tris, pH 8.0, 13 mM EDTA). The pellets were then resuspended in 125  $\mu$ l of ice-cold distilled water, sulfuric acid was added to a final concentration of 0.4 N. Subsequently, lysates were incubated on ice for 1 h followed by centrifugation at 10,000  $\times$  g for 5 min. The supernatants were precipitated with  $\times$ 10 volumes of acetone at -20 °C overnight. The precipitated histones were collected by centrifugation, dried under vacuum, and resuspended in distilled water. Protein concentration was measured by using BCA protein assay reagent.

**Immunoblot Analysis of Acetylated Histone H4**—Equal amounts of histones (10  $\mu$ g) were electrophoresed on an 18% SDS-PAGE gel and blotted on nitrocellulose membranes or stained with Coomassie Blue. Blots were blocked by incubating with PBS containing 3% dry milk (PBS-MLK) for 20 min at room temperature, and then probed with

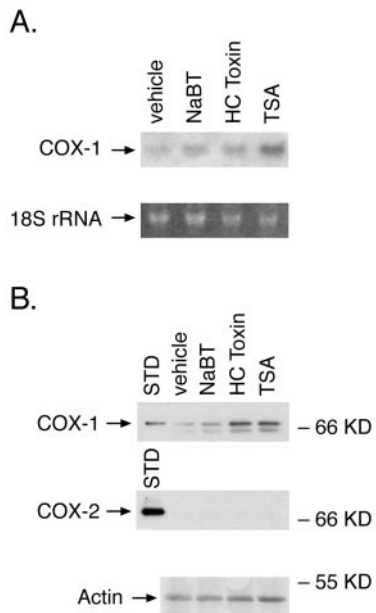
anti-acetylated histone H4 anti-rabbit antibody (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:2000 in PBS-MLK overnight at 4 °C. After washing, blots were incubated with anti-rabbit secondary antibody at a dilution of 1:3000 in PBS-MLK for 1.5 h, and detected by an ECL kit. For Coomassie Blue staining, gels were incubated for 1 h in staining buffer (0.25% Coomassie Blue, 10% acetic acid, and 40% methanol), and then destained with repeated changes of acid/methanol.

**Northern Blot Analysis**—Total RNA from the semiconfluent cells on 100-mm diameter dishes was extracted using TRI Reagent (Sigma) according to the manufacturer's instruction. RNA samples (10  $\mu$ g/lane) were separated by electrophoresis in a 0.66 M formaldehyde, 1% agarose gel. The RNA was transferred in 10  $\times$  SSC onto nylon membrane (Hybond-N<sup>+</sup>, Amersham Biosciences) by capillary action and UV cross-linked with a Stratilinker UV light source (Stratagene, La Jolla, CA). Human COX-1 was purchased from Oxford. The cDNA probe was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Prime-It-II random prime kit (Stratagene). Blots were prehybridized in Rapid-hyb buffer (Amersham Biosciences) at 60 °C for 2 h, followed by hybridization at 60 °C overnight. The blots were then washed once in 2  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS at room temperature and then twice in 0.1  $\times$  SSC, 0.1% SDS at 60 °C. The membrane was exposed to Hyperfilm (Amersham Biosciences) for 24 h.

**Reverse Transcriptase-PCR and Sequence Analysis**—First-strand cDNA was generated from 1  $\mu$ g of total RNA using Advantage reverse transcription for polymerase chain reaction (PCR) kit (CLONTECH, Palo Alto, CA). The reaction solution was diluted to final volume of 100  $\mu$ l by adding of diethyl pyrocarbonate-treated water. Ten  $\mu$ l of the cDNA solution were used as PCR. PCR mixture consisted of 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 2.0 units of *Taq* DNA polymerase (Promega, Madison, WI), and 0.4  $\mu$ M each of the following primers, which were specific for full-length human COX-1 (33). Primers for COX-1 (5'-GCACCCAGCAGCCGCGCCATGA-3', 5'-GCTGCTTTCCTGCCCTCAGAGCTC-3') were used. The samples for COX-1 were then subjected to a first denaturation (4 min at 94 °C), and 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 70 °C), and extension (2 min at 72 °C), followed by a final extension at 72 °C for 7 min. PCR product of 1.8-kb fragment was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced by ABI PRISM dRhodamine Terminator Cycle Sequencing Ready kit (PerkinElmer Life Science, Foster City, CA).

**Analysis of Arachidonic Acid Metabolite Activity**—1  $\times$  10<sup>5</sup> NHA cells were plated in each well of a 24-well plate and incubated for 24 h. The cells were treated with or without 200 ng/ml TSA in astrocyte growth medium with 5% FBS. After 48 h, the medium was replaced with astrocyte basal medium from Clonetics without FBS containing 10  $\mu$ M arachidonic acid (Cayman) for 1 h. The supernatants were harvested, and then samples were stored at -80 °C until further use. Concentrations of PGE<sub>2</sub> in cell culture supernatants were determined by enzyme immunoassay using a PGE<sub>2</sub> monoclonal enzyme immunoassay (EIA) kit (Cayman) according to the manufacturer's instructions. The lower limit of sensitivity for detection of PGE<sub>2</sub> was 31.3 pg/ml in our experimental condition. The results are expressed as nanograms/ml per 10<sup>5</sup> cells.

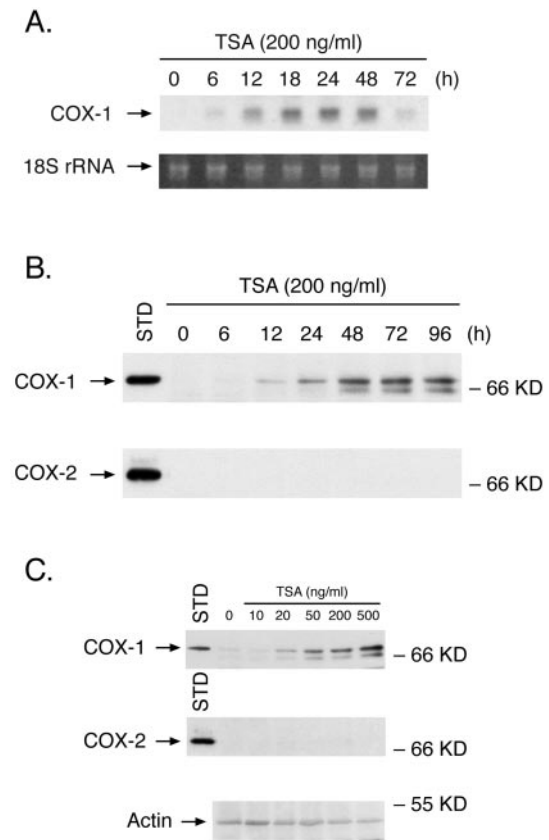
**Transient Transfection and Luciferase Assay**—COX-1 promoter/reporter plasmids were constructed as previously described (34). In this study, the 5'-untranslated region of COX-1, from -1960 to +115, from -609 to +115, from -430 to +115, and from -2 to +115 were generated by PCR amplification using genomic DNA from NHA cells. PCR was performed using Expand High Fidelity PCR system (Roche Molecular Diagnostics Corp., Indianapolis, IN) according to the manufacturer's instructions. Nucleotides illustrated here are relative to the transcription initiation site that is +1 (GenBank™ accession number AF440204). The following primers were used: upstream primers, from -1960, 5'-ACCGGTACCGAGCCAGAAG-3'; from -609, 5'-GATGGTACCCTGAGGGCT-3'; from -430, 5'-CTGGGTACCCTGTCTGAGGA-3'; from -2, 5'-GCGGGTACCCGAGGTGACA-3'; downstream primer, from +115, 5'-TGGAAGCTTGACGCAGAGT-3'. All of upstream PCR primers contained *Kpn*I recognition site, and downstream primer contained *Hind*III recognition site. The PCR products were purified from agarose gel, digested, and cloned into the pGL3-Basic vector (Promega). In pGL3-2/+115, the Sp1 putative binding sites (+25/+31) were mutated using the QuikChange Site-directed Mutagenesis kit (Stratagene). For the point mutation of Sp1-binding sites, the following primers were used: MUT sense, 5'-CTGGAGGGAGGAGCGGTTTTAGAGCCGGGGGAAGGG-3'; and MUT antisense, 5'-CCCTTCCCCCGGCTCTAAACCGCTCCTCCCTCCAG-3'. Each construct was confirmed by DNA sequencing. For transfection experiment, cells were plated in 12-well plates at a density of 2  $\times$  10<sup>5</sup> cell/well and incubated in astrocyte growth medium containing FBS for 24 h. After washing the cells with PBS, 500



**FIG. 1. Induction of COX-1 in NHA cells by treatment with HDAC inhibitors.** Semiconfluent NHA cells were treated with HDAC inhibitors (1 mM NaBT, 20 nM HCT, 200 ng/ml TSA) or vehicle for 48 h. *A*, the total RNA (10  $\mu$ g) were examined by Northern blot analysis using a probe for human COX-1. The mRNA for COX-1 is 2.7 kb. Equal loading of 18 S RNA was visualized under UV light with ethidium bromide staining of the gel (*bottom panel*). *B*, cells were incubated with HDAC inhibitors for 48 h. Equal amounts (20  $\mu$ g) of total protein were loaded to each lane and separated on an 8% SDS-PAGE. After transfer to nitrocellulose membrane, the immunoblot were probed with antibodies specific for COX-1, COX-2, and actin, and detected by the ECL system.

ng of luciferase reporter construct was transfected using 2.5  $\mu$ l of LipofectAMINE (Invitrogen) in 500  $\mu$ l of astrocyte basal medium without FBS at 37  $^{\circ}$ C for 4 h. The medium was replaced and the cells were incubated for an additional 48 h with HDAC inhibitors (1 mM NaBT, 200 ng/ml TSA, or 20 nM HC toxin) or vehicle (0.1% Me<sub>2</sub>SO). The cells were harvested using 250  $\mu$ l of passive lysis buffer (Promega) for the luciferase assay and quantification of protein content. Twenty  $\mu$ l of cell lysate was used for firefly luciferase assays. Relative light unit values were measured with firefly assay reagent (Promega) and a model TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA), and protein concentrations from corresponding cell lysate were measured using the BCA protein assays. Data were expressed as relative light units per microgram of protein. All luciferase assays were carried out in triplicate.

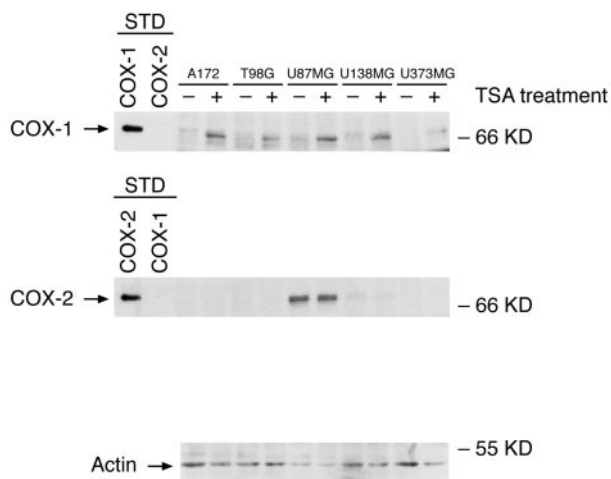
**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared as described previously (35) and stored at  $-80^{\circ}$ C. Protein concentration was determined using BCA protein assays. An oligonucleotide probe was synthesized based on the sequence of the promoter region of COX-1 including the known Sp1-binding site: WT-COX1-Sp1, GAGGAGCGGGGTGGAGC-CGGGGGAA (nucleotides +17 to +42). Each complementary oligonucleotide was annealed at a concentration of 250 mM in 250 mM Tris (pH 7.8) at 95  $^{\circ}$ C for 15 min and then cooled slowly to room temperature. Probe was labeled using [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The Sp1 GELSHIFT kit was obtained from Geneka Biotechnology (Montreal, Quebec, Canada) and the procedure for reaction between the probe and 4  $\mu$ g of nuclear extracts was followed according to the manufacturer's instruction. Briefly, nuclear extracts were incubated with or without antibodies in 4  $\mu$ l of binding buffer B and 2  $\mu$ l of stabilizing solution D (total volume was 16  $\mu$ l) at 4  $^{\circ}$ C for 20 min, and each mixture was incubated after adding 200,000 cpm of labeled probe in 2  $\mu$ l of binding buffer C and 1  $\mu$ l of stabilizing solution D (total volume was 24  $\mu$ l) at 4  $^{\circ}$ C for 20 min. For supershift assay, 4  $\mu$ g of antibodies for Sp1, Sp2, Sp3, and Sp4 (Santa Cruz) were used. Samples were then resolved in 4% nondenaturing polyacrylamide (38:2) gel, at 4  $^{\circ}$ C in 1  $\times$  TGE buffer. Gels were dried under vacuum and exposed to x-ray film. Caco-2 cell nuclear extracts (Geneka Biotechnology) were used as a positive control.



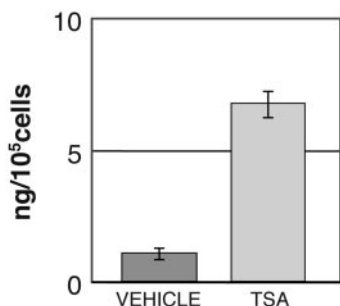
**FIG. 2. Induction of COX-1 in NHA cells by treatment with TSA.** Semiconfluent NHA cells were treated with 200 ng/ml TSA for the times indicated. *A*, total RNA was extracted and analyzed by Northern hybridization using probe for a human COX-1. *B*, proteins were extracted from the cells, and examined by Western blot analysis using antibodies specific for COX-1, COX-2, and actin. *C*, the semiconfluent NHA cells were treated with various concentrations of TSA for 48 h. Equal amounts (20  $\mu$ g) of total protein were examined by Western blot analysis using antibodies specific for COX-1.

## RESULTS

**Induction of COX-1 in NHA Cells after Treatment with HDAC Inhibitors**—To examine the effects of several HDAC inhibitors on the expression of lipid metabolizing enzymes in NHA cells, cells were treated with NaBT, HC toxin, and TSA for various times and doses. Levels of 15-LO-1, COX-1, and COX-2 protein and mRNA were analyzed by Western blot and Northern blot analysis, respectively. We did not detect the expression levels of 15-LO-1 and COX-2 in HDAC inhibitors or vehicle-treated cells (data not shown) as observed in colorectal carcinoma cells (19). However, treatment with HDCA inhibitors increased levels of COX-1 mRNA (2.7 kb) and protein (67 kDa) (Fig. 1, *A* and *B*). TSA treatment enhanced COX-1 mRNA and protein in a time-dependent manner (Fig. 2, *A* and *B*). The COX-1 mRNA and protein was detected at 12 h after treatment and the level was maximal at 24 and 48 h, respectively. Furthermore, the induction of COX-1 appeared in a dose-dependent manner in NHA cells. NHA cells were treated with various concentrations of TSA ranging from 0 (vehicle) to 500 ng/ml (Fig. 2*C*). TSA increased COX-1 protein even at 20 ng/ml. To confirm that COX-1 expression was induced, RNA was isolated from TSA-treated cell and reverse transcriptase-PCR was performed with specific primers for human COX-1. After PCR, the 1.8-kb fragment was cloned into the TA vector (3.9-kb) and partially sequenced (33). The sequence was identical to the reported human COX-1 (data not shown). These results indicate that HDAC inhibitors altered the expression of COX-1 rather than the induction of COX-2 and 15-LO-1 in NHA cells.



**FIG. 3. Induction of COX-1 protein in glioma tumor cells by treatment with HDAC inhibitors.** The semiconfluent glioma cell lines (A172, T98G, U87MG, U138MG, and U373MG) were treated with 200 ng/ml TSA or vehicle for 48 h. Equal amounts (20  $\mu$ g) of total protein isolated from cells were separated on an 8% PAGE for analysis of COX-1, COX-2, and actin expression.

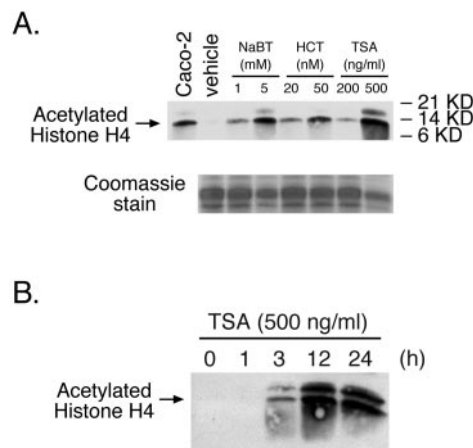


**FIG. 4. PGE<sub>2</sub> production by NHA cells treated with TSA.** Cells were treated for 48 h with 200 ng/ml TSA or vehicle. Cells were washed and then incubated in the presence of 10  $\mu$ M arachidonic acid for 1 h. The concentration of PGE<sub>2</sub> in the medium was determined by an enzyme immunoassay as described under "Experimental Procedures."

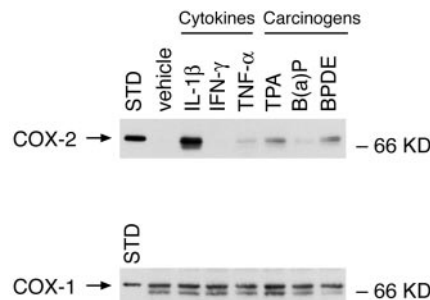
**Expression of COX-1 in Glioma Cell Lines after Treatment with HDAC Inhibitors**—NHA cells are normal glial cells. Then, to ascertain whether treatment with HDAC inhibitors stimulate COX-1 expression in transformed glial cells, we examined five glioma (glial tumor) cell lines, A172, T98G, U87MG, U138MG, and U373MG. Induction of COX-1 protein was observed after TSA treatment in all five cell lines (Fig. 3), suggesting that COX-1 is an inducible gene in glial-derived cells, and that regulation of COX-1 expression in glial-derived cells appears to be controlled by a unique mechanism associated with histone acetylation. COX-2 protein expression was not detected in these glioma cell lines except U87MG cells. TSA did not alter COX-2 protein expression in U87MG cells.

**Metabolic Activity in TSA-treated NHA Cells**—NHA cells were treated with or without 200 ng/ml TSA for 48 h, the medium was replaced with fresh serum-free medium, containing 10  $\mu$ M exogenous arachidonic acid. After 1 h incubation, the concentrations of PGE<sub>2</sub> in the cell culture supernatants were determined using an EIA. PGE<sub>2</sub> production increased from 1.1 to 6.8 ng/10<sup>5</sup> cells by TSA treatment (Fig. 4). Thus, treatment of NHA cells with HDAC increased not only COX-1 expression but also its enzymatic activity.

**Immunoblot Analysis of Acetylated Histone H4**—To confirm if HDAC inhibitors increase histone acetylation, we examined the effects of HDAC inhibitors on the acetylation of histone H4 protein in NHA cells. As shown in Fig. 5A, treatment with HDAC inhibitors caused a dramatic increase in acetylated hi-



**FIG. 5. Histone H4 acetylation in NHA cells.** A, histone proteins were isolated from the semiconfluent NHA cells treated with the various HDAC inhibitors or vehicles for 48 h as described under "Experimental Procedures." Ten  $\mu$ g of histone proteins were separated on 18% SDS-PAGE and subjected to analysis of acetylated H4 by Western analysis using an anti-acetylated H4 antibody or stained with Coomassie Blue. Histone fraction isolated from NaBT-treated Caco-2 cells (human colorectal carcinoma cell line) served as positive control. B, the semiconfluent NHA cells were treated with 500 ng/ml TSA for the times indicated and the level of acetylated histone H4 was examined by Western analysis as described above.

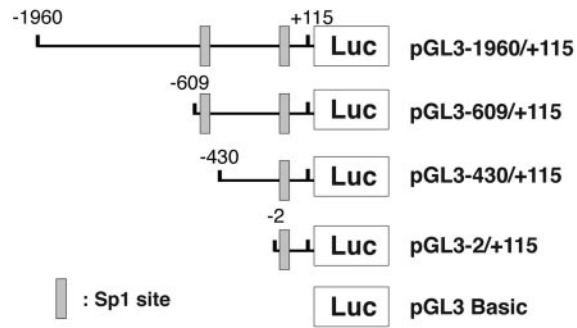


**FIG. 6. Induction of COX-2 protein in NHA cells by treatment with cytokines and carcinogens.** The semiconfluent NHA cells were treated with cytokines (10 ng/ml interleukin 1- $\beta$ , 1 ng/ml IFN- $\gamma$ , 10 ng/ml tumor necrosis factor- $\alpha$ ), carcinogens (100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate, 10  $\mu$ M benzo(a)pyrene, 1  $\mu$ M benzo(a)pyrene diol-epoxide), or vehicle for 24 h. After the cells were lysed, the isolated proteins (20  $\mu$ g) were examined by Western blot analysis using antibodies specific for COX-1 and COX-2.

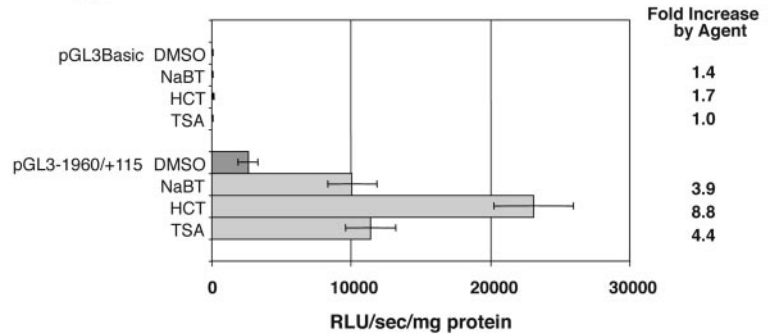
stone H4 protein and this increase occurred in a dose-dependent fashion. Histone fractions isolated from NaBT-treated Caco-2 cells, a human colorectal carcinoma cell line, served as a positive control (23). Thus HDAC inhibitors induce COX-1 expression and increase the hyperacetylated forms of histone H4 in the NHA cells. We next determined the time course for the increase in histone acetylation. As shown in Fig. 5B, acetylation was detected as early as 3 h following treatment with a peak around 12 h. Thus the increase in acetylation of histone preceded that of COX-1 expression.

**Effect of Cytokines on COX-1 and COX-2 Expression**—Many inflammatory cytokines increase the expression of COX-2 in cells with little or no effect on the expression of COX-1 (25, 36). Thus we decided to examine the effects of cytokines on COX-1 and COX-2 protein expression of the NHA cells. Cells were incubated with the cytokines for 24 h. Once the cells were harvested, the protein was analyzed by Western analysis. None of the cytokines tested altered the expression of COX-1 but interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  increased COX-2 expression (Fig. 6). In addition, incubation with the carcinogens (12-*O*-tetradecanoylphorbol-13-acetate, benzo(a)pyrene,

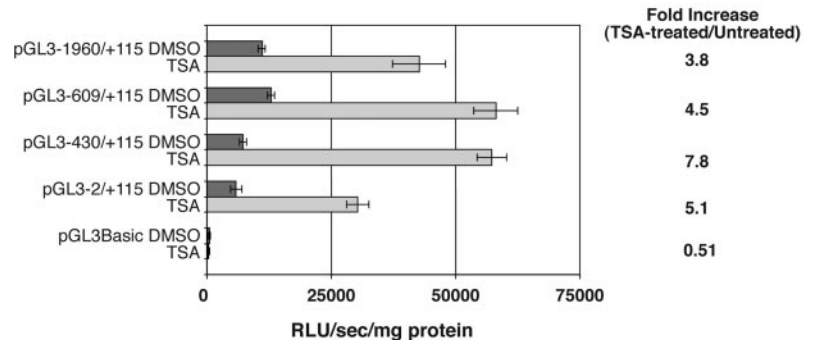
A.



B.



C.

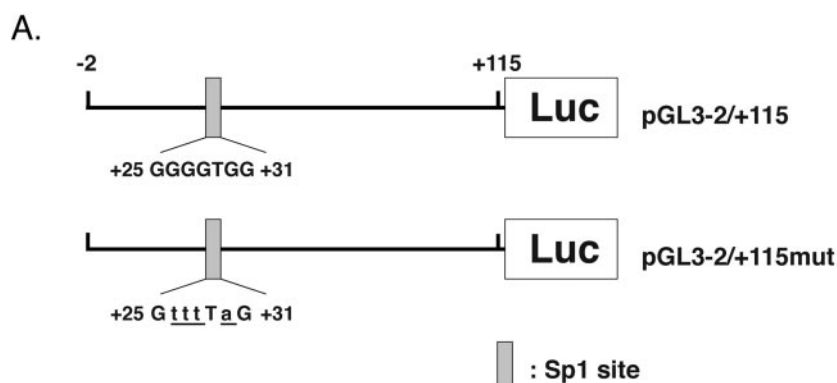


**FIG. 7. HDAC inhibitors activate transcription from COX-1 promoter.** A, the indicated fragments of human COX-1 promoter regions were constructed in pGL3Basic luciferase (LUC) reporter plasmid as pGL3-1960/+115, pGL3-609/+115, pGL3-430/+115, and pGL3-2/+115, respectively. B, the pGL3-1960/+115 plasmid was transiently transfected into NHA cells, and luciferase activities were analyzed after 48 h treatment with vehicle, 1 mM NaBT, 200 ng/ml TSA, or 20 nM HC toxin. C, cells were transiently transfected with the constructed plasmids pGL3-1960/+115, pGL3-609/+115, pGL3-430/+115, pGL3-2/+115, and luciferase activities were measured after 48 h treatment with vehicle or 200 ng/ml TSA. Data were normalized by protein concentration and expressed as relative light unit (RLU) per microgram of protein. Data shown are mean  $\pm$  S.E. ( $n = 3$ ). The fold increase in promoter activity by HDAC inhibitors is shown on the right.

and its more potent metabolite benzo(a)pyrene diol-epoxide) increased the expression of COX-2 without altering COX-1 expression (Fig. 6). Thus, the change of COX-1 and COX-2 expression in NHA cells after cytokine treatment is in agreement with previous studies in other cells and supports the notion that HDAC inhibitors regulate COX-1.

**Analysis of the Promoter Activity of the 5'-Regulatory Region of the Human COX-1 Gene**—We isolated the promoter region of the COX-1 gene (−1960 to +115) from genomic DNA of NHA cells, and cloned it into pGL3Basic upstream of the luciferase gene (Fig. 7A). The effect of HDAC inhibitors on the promoter activity of luciferase gene expression of pGL3-1960/+115 was examined in NHA cells. Following a 48-h exposure to HDAC inhibitors, luciferase activity was assayed. The NHA cells transfected with pGL3-1960/+115 reporter construct showed 3.9-, 8.8-, and 4.4-fold activation with NaBT, HCT, and TSA, respectively. On the other hand, HDAC inhibitors showed a little activation (<1.7-fold) on pGL3Basic reporter construct-transfected cells (Fig. 7B). To further determine the location of the transcription binding site(s) regulated by HDAC inhibitors, 4 different constructs were prepared with different promoter lengths, this resulted in the removal of each of the two Sp1-

binding sites located in the COX-1 promoter as shown in Fig. 8A. These sites were chosen because previous work indicated that two Sp1-binding sites were involved in basal endothelial COX-1 promoter activity (34), and Sp1-binding sites were involved in the transcriptional activation of some genes in response to HDAC inhibitors (14, 16, 37). The regulation of the COX-1 gene by HDAC inhibitors was analyzed by comparing the transcriptional activity of different promoter flanking regions in NHA cells with or without TSA treatment (Fig. 7C). The fold increase in the promoter activity by TSA treatment was not decreased by a progressive 5'-deletion. Deletion of distal Sp1-binding site located at −475 to −469 did not alter the luciferase activity. However, deletion of the proximal Sp1-binding site at +25 to +31 eliminated TSA stimulation of the activity. This finding suggests that the proximal Sp1-binding site located near the transcription initiation site is required for the regulation of COX-1 expression by HDAC inhibitors. We then generated mutant constructs having mutation in the proximal Sp1-binding site (Fig. 8A). Luciferase activities were measured in the cells after treatment with or without TSA (Fig. 8B). The basal activity of pGL3-2/+115mut was reduced to 14% of that of pGL3-2/+115. The activation by TSA in pGL3-2/



**FIG. 8. Mutation of the Sp1 site present in the COX-1 promoter in NHA cells.** A, the Sp1-binding site of pGL3-2/+115 was mutated by site-directed mutagenesis, and the mutant promoter/reporter plasmid designated as pGL3-2/+115mut. The *underlined lowercase* nucleotides represent mutations. B, NHA cells were transiently transfected with pGL3-2/+115 or pGL3-2/+115mut and then treated or 48 h with vehicle or 200 ng/ml TSA. Luciferase activities were measured and expressed as relative light unit (RLU) per microgram of protein. Data shown are mean  $\pm$  S.E. ( $n = 3$ ). The fold increase in promoter activity by TSA treatment is shown on the *right*.

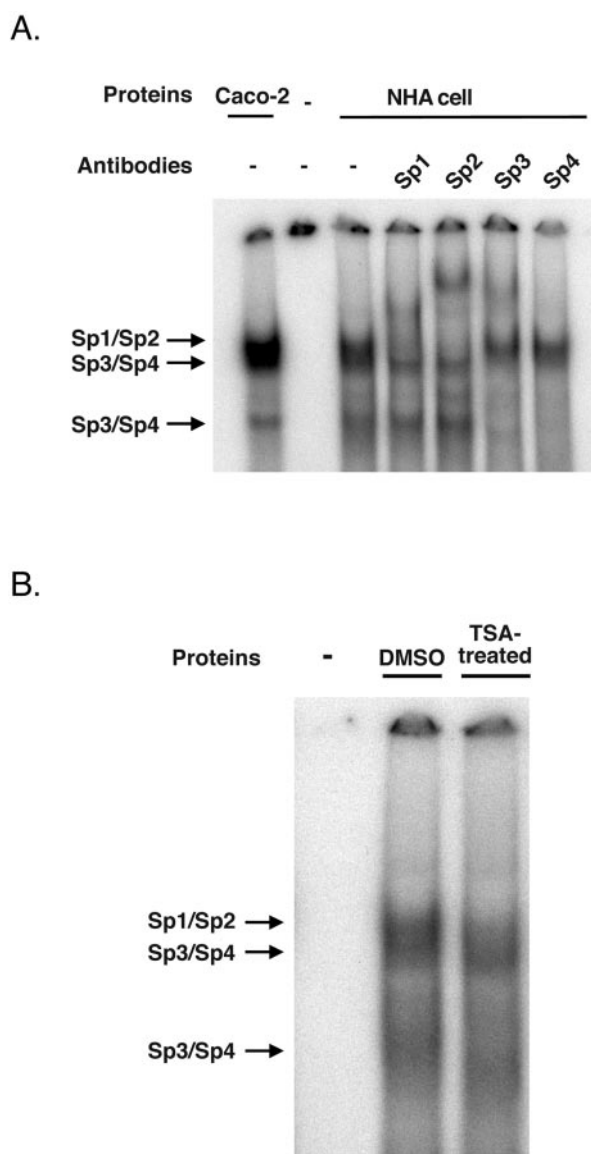
+115mut resulted in a further decrease in activity and was 48% of its basal activity. This compares to 3.0-fold activation in wild type pGL3-2/+115. These findings strongly support the conclusion that TSA activates the COX-1 promoter through the effect at the proximal Sp1-binding site.

**Complex Formation between Labeled Oligonucleotide and Nuclear Extracts**—To further examine the involvement of Sp1 in the TSA response, EMSA was performed using nuclear extract from NHA cell with or without TSA treatment. Nuclear extracts from Caco-2 cells were used as a positive control for Sp1 binding. End-labeled DNA probes (COX-1 promoter: nucleotides +17 to +42) containing the putative Sp1-binding site were combined with nuclear extracts. As shown in Fig. 9A, two major DNA-protein complexes were detected. These complexes were diminished by addition of an excess of unlabeled homogenous oligonucleotide (data not shown). Incubation of the extract with Sp1, -2, -3, and -4 antibodies resulted in a supershift band with Sp1, -2, and -3 but not Sp4. These data support the conclusion for the binding of Sp family of proteins to the Sp1-binding site in COX-1 promoter in NHA cells. We next examined if treatment with TSA altered the binding of Sp isoforms with COX-1 promoter. Treatment with TSA did not alter the mobility pattern but did decrease the intensity of bands (Fig. 9B), suggesting that TSA mediates COX-1 promoter activity by a mechanism other than directly altering the DNA binding activities of Sp1.

#### DISCUSSION

In this study, we report that COX-1 mRNA and protein is induced by HDAC inhibitors in a concentration- and time-dependent manner in NHA cells and in 5 different glial tumor cell lines. The human COX-1 promoter contains two Sp1-binding sites and the proximal Sp1-binding site is required for the regulation of expression by HDAC inhibitors. This conclusion is supported by the following results: an increase in histone acetylation is observed after treatment with TSA, a HDAC inhibitor; deletion of the Sp1-binding site from a COX-1 promoter luciferase construct abolishes HDAC induced promoter activity; mutation of the Sp1-binding site in COX-1 promoter abolishes activity, and finally the Sp1 family of transcription factors specifically bind to the Sp1-binding site present in the COX-1 promoter.

These conclusions are consistent with the effects of HDCA inhibitors on the activation of other genes (13, 15). Recent studies have reported that the GC box in the promoter region of p21<sup>WAF1/Cip1</sup> is important for basal and TSA-induced promoter activity and that Sp1 and Sp3 are activators of this GC-box (14, 16). Other studies have reported that HDAC inhibitors activate transcription from the G $\alpha_{12}$  gene promoter by Sp1 binding in differentiating K562 cells (37). HDAC inhibitors release an inhibitory constraint on Sp1, which results in association with accessory protein to effect gene transcription. These findings support the hypothesis that Sp1-binding sites are involved in



**FIG. 9. Complex formation between labeled oligonucleotide and nuclear extracts.** *A*, nuclear extracts were prepared from Caco-2 cells, and NHA cells. These were subjected to EMSA using end-labeled oligonucleotide containing the sequence of the putative Sp1-binding site between +17 and +42 in the COX-1 promoter as a probe. Nuclear extracts from Caco-2 cells were used for positive control as Sp1 transcriptional factor. Specific antibodies for Sp1, -2, -3, and -4 were used as indicated. An arrow shows the band of Sp1, -2, -3, and -4 proteins and labeled probes complex. *B*, nuclear extracts were prepared from NHA cells treated with Me<sub>2</sub>SO alone or TSA at 200 ng/ml for 24 h, and subjected to EMSA as described above.

the transcriptional activation of genes in response to HDAC inhibitors. On the other hand, Doetzlhofer *et al.* (12) reported that Sp1 could be a target for HDAC1-mediated transcriptional repression.

Sp1 plays a key role in the activation of a large number of genes, including housekeeping and cell cycle-regulated genes, containing upstream "GC Box" promoter elements (38). Furthermore, Sp1 is implicated in important regulatory functions during cell development, differentiation, and apoptosis (14), and contributes to the induction of several genes such as interleukin-1 $\beta$  (39), p15<sup>INK4B</sup> (40), and TNFR-II (41). The 5'-flanking region of the COX-1 gene has multiple transcriptional start sites, does not possess a TATA or CAAT box, and is GC rich. The Sp1-binding sites of COX-1 promoter region activate the basal COX-1 gene transcription (34). Our data also indicated that

this Sp1 site is involved in COX-1 transcription in NHA cells. However, its DNA-protein complex as measured by EMSA was not change by TSA treatment, which suggest that TSA induced activation of COX-1 promoter activity is not related to changes in DNA binding activities of Sp proteins. This finding is similar to lipopolysaccharide stimulation of the interleukin-10 promoter in macrophage cells (42). Additionally, transcriptional activation requires the Sp1-binding site in the promoter but is not dependent on DNA binding. Therefore, modulation of Sp1 proteins by phosphorylation or glycosylation, for example, may provide an explanation for the activation of COX-1 promoter by HDAC inhibitors.

Histone acetylation is associated with transcriptional activity in eukaryotic cells (5–7). Acetylation occurs at lysine residues on the amino-terminal tails of histones, resulting in alteration of nucleosomal conformation, thus increasing the accessibility of transcriptional regulatory proteins to chromatin templates (5, 7), and subsequent transcription. Histone acetyltransferase promotes transcription, while HDAC should act as a repressor. Generally speaking, inhibition of HDAC results in acetylation of histone protein. Furthermore, inhibition of HDAC is known to affect a variety of biological processes, such as the induction of differentiation, cell cycle arrest, and apoptotic cell death. An increase in the level of histone acetylation and subsequent relaxation of chromatin at the sites of active transcription is thought to be one mechanism by which HDAC inhibitor activates gene expression.

COX-1 is constitutively expressed in a wide variety of tissues, while COX-2 is a highly inducible gene that is expressed in response to a variety of proinflammatory agents, cytokines, growth factors, and tumor promoters. But there are some reports where the expression of COX-1 is also regulated (30, 36, 43–47). For example, 12-*O*-tetradecanoylphorbol-13-acetate induces COX-1 expression in human umbilical vein endothelial cells (47) and human monocytic leukemia THP-1 cells (46), and nerve growth factor induces COX-1 in the rat pheochromocytoma PC12 cell lines (44). COX-1 is also induced in mouse osteoblastic MC3T3 cells treated with basic fibroblastic growth factor (36). Rioux *et al.* (45) demonstrated that tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, induced expression of COX-1 in the human lymphoma U937 cells.

Little is known about the biological function and regulation of COX-1 expression in the human brain. It is known, however, that COX-1 expression is up-regulated during brain development (29). COX-1 is also up-regulated by retinoic acid during neuronal differentiation of neuroblastoma cell lines (30). Yermakova *et al.* (28) reported a possible relationship between COX-1 expression and Alzheimer's disease. They found COX-1 immunopositive microglia in association with  $\beta$ -amyloid plaques, and the density of COX-1 immunopositive microglia in fusiform cortex were increased, indicating the possibility that COX-1 may contribute to central nervous system pathology.

In conclusion, the results presented here support the hypothesis for the regulation of COX-1 expression by a unique mechanism associated with histone acetylation in glial cells. The regulation of COX-1 expression by histone acetylation occurs via an Sp1-binding site located in the COX-1 promoter. Further studies are required to fully elucidate the precise mechanism.

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