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Endoplasmic Reticulum Stress Stimulates the Expression of Cyclooxygenase-2 through Activation of NF-κB and pp38 Mitogen-activated Protein Kinase*

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Expression of mutant proteins or viral infection may interfere with proper protein folding activity in the endoplasmic reticulum (ER). Several pathways that maintain cellular homeostasis were activated in response to these ER disturbances. Here we investigated which of these ER stress-activated pathways induce COX-2 and potentially oncogenesis. Tunicamycin and brefeldin A, two ER stress inducers, increased the expression of COX-2 in ML-1 or MCF-7 cells. Nuclear translocation of NF-κB and activation of pp38 MAPK were observed during ER stress. IκBα kinase inhibitor Bay 11-7082 or IκBα kinase dominant negative mutant significantly inhibited the induction of COX-2. pp38 MAPK inhibitor SB203580 or eIF2α phosphorylation inhibitor 2-aminopurine attenuated the nuclear NF-κB DNA binding activity and COX-2 induction. Expression of mutant hepatitis B virus (HBV) large surface proteins, inducers of ER stress, enhanced the expression of COX-2 in ML-1 and HuH-7 cells. Transgenic mice showed higher expression of COX-2 protein in liver and kidney tissue expressing mutant HBV large surface protein *in vivo*. Similarly, increased expression of COX-2 mRNA was observed in human hepatocellular carcinoma tissue expressing mutant HBV large surface proteins. In ML-1 cells expressing mutant HBV large surface protein, anchorage-independent growth was enhanced, and the enhancement was abolished by the addition of specific COX-2 inhibitors. Thus, ER stress due either to expression of viral surface proteins or drugs can stimulate the expression of COX-2 through the NF-κB and pp38 kinase pathways. Our results provide important insights into cellular carcinogenesis associated with latent endoplasmic reticulum stress.

The endoplasmic reticulum (ER)[1] stress response is a mechanism by which cells respond to excess unfolded proteins in the ER (1–5). The ER stress response is suggested to contribute to several types of human disease, including degenerative neuronal disorders (5–7) and type II diabetes (8, 9). ER stress response is induced by overexpression of exogenous membrane or secretory proteins, such as virus gene-encoded proteins (10–12). Hepatitis C virus and Japanese encephalitis virus infection initiate endoplasmic reticulum stress (13–15). Drugs that perturb ER function (*e.g.* glycosylation inhibitors such as tunicamycin (TM) or disulfide bond reducing agents such as 2-mercaptoethanol) can be used to study the following two types of ER signal pathways (1): the unfolding protein response (UPR), and the ER-overloaded response (EOR) pathway.

The UPR pathway has three components in mammalian cells: basic leucine zipper transcription factor ATF6, IRE1 RNA-processing enzyme, and ER localized kinase (PERK). ATF6 is synthesized as an ER transmembrane protein and is cleaved to generate cytosolic transcription factors that migrate to the nucleus. ATF6 cooperates with transcription factor NF-Y to bind mammalian ER stress-responsive elements (ERSE) to UPR-responsive gene promoters, such as *GRP78* (16–18). Mammalian IRE1 ribonuclease is activated by accumulation of unfolded protein in the endoplasmic reticulum. By removing a 26-nucleotide intron from XBP-1 mRNA, active IRE1 produces a novel XBP-1 mRNA encoding a transcription factor that can act via ERSE to activate the transcription of many UPR-responsive genes (19–22). In addition to activation of the ERSE-related transcription pattern, ER stress also alters the translational pattern through PERK. The C-terminal cytoplasmic kinase domain of PERK can directly phosphorylate translation factor eIF2α and cause translational repression in response to an upstream ER stress signal (23, 24).

The EOR pathway triggers the activation of transcription factor NF-κB (1, 14, 25–28). The activation of NF-κB has been suggested to require the release of calcium from the ER and the production of reactive oxygen species (28). STAT3 transcription factor may mediate part of the activation of the JAK-STAT

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[1] The abbreviations used are: ER, endoplasmic reticulum; MAPK, mitogen-activated protein kinase; HBV, hepatitis B virus; COX, cyclooxygenase; TM, tunicamycin; UPR, unfolding protein response; EOR, ER-overloaded response; ESRE, ER stress-responsive elements; JNK, c-Jun N-terminal kinase; PDTC, pyrrolidine dithiocarbamate; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; EMSA, electrophoretic mobility shift assay; PG, prostaglandin; 2-AP, 2-aminopurine; pon-A, ponasterone A; LMB, leptomycin B; ERK, extracellular signal-regulated kinase; PERK, phosphorylated ERK; eIF, eukaryotic initiation factor; EIA, enzyme immunoassay; HBsAg, hepatitis B surface antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

pathway by reactive oxygen species (14). Activation of NF-κB is also reported to be mediated via tumor necrosis factor receptor-associated factor 2 and c-Jun N-terminal kinase (JNK) (29, 30). Although the EOR and UPR pathways are distinct, they are related. Overexpression of IRE1 can activate NF-κB, and dominant negative IRE1 can inhibit activation of NF-κB (29). Phosphorylation of the α subunit of eukaryotic initiation factor 2 by PERK is required for activation of NF-κB in response to endoplasmic reticulum stress (31). Activation of NF-κB by ER stress leads to induction of many cellular genes that are largely anti-apoptotic in function. Latent long term expression of many viral surface proteins in mammalian cells may lead to cellular carcinogenesis, which in turn may be partly associated with the ER stress induced by these proteins (12, 14).

Overexpression of cyclooxygenase (COX)-2 has been found in many types of cancer and was linked to disease progression and drug resistance (32–38). Overexpression of COX-2 is sufficient to induce tumorigenesis or sensitize mouse skin for carcinogenesis (39, 40). Cyclooxygenase-2 expression is regulated through multiple pathways including NF-κB, C/EBP transcription factors, and mitogen-activated protein kinases (41–46). Induction of COX-2 mRNA is regulated by NF-κB in macrophages (41–44). The expression of COX-2 is correlated with the increase of NF-κB activity, and induction of COX-2 by interleukin-1 is mediated partly by NF-κB in colorectal cancer cells (45, 46).

Because NF-κB is induced by ER stress, and NF-κB can regulate the expression of COX-2, we hypothesized that ER stress may induce the expression of COX-2 and regulate cellular homeostasis. In this report, we demonstrated ER stress induced by tunicamycin (TM) and brefeldin A leads to increased expression of COX-2. The induction of COX-2 was mediated through NF-κB and p38 pathways. Furthermore, ER stress induced by expression of hepatitis B virus surface protein also enhanced the expression of COX-2 *in vitro*. Mutant hepatitis B virus surface protein expression induced the *in vivo* expression of COX-2 in transgenic mice. Finally, expression of mutant HBV large surface proteins enhanced anchorage-independent growth of hepatocytes, which is dependent on the induction of COX-2.

EXPERIMENTAL PROCEDURES

Chemicals and Kits—Tunicamycin, brefeldin A, actinomycin D, pepstatin, sodium orthovanadate, leupeptin, dithiothreitol, ethidium bromide, SDS, Bay 11-7082, pyrrolidine dithiocarbamate (PDTC), and leptomycin B were products of Sigma. PD98059, SD203580, and JNK inhibitor II were purchased from Calbiochem. Ponasterone A was from Stratagene (La Jolla, CA). [γ-^{32}P]ATP (6000 Ci/mmol) and ECL Western blot detection system were from Amersham Biosciences. The prostaglandin E_2 EIA kit was from Cayman Chemical (Ann Arbor, MI). HBsAg enzyme-linked immunosorbent assay (ELISA) kit was from General Biological Corp. (Taipei, Taiwan). RT-PCR reagent and G418 was from Promega (Madison, WI). The NE-PER nuclear and cytoplasmic extraction reagents kit and Micro BCA™ protein assay reagent kit were from Pierce. Anti-COX-2 and anti-GRP78 were purchased from Transduction Laboratories. Anti-p38, anti-phospho-p38, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-eIF2α, and antiphospho-eIF2α antibodies were from Cell Signaling (Beverly, MA). Anti-IκBα, anti-p65, and anti-RelB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p50 was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-α-tubulin was from MDBio (Frederick, MD). Anti-β-actin was from Chemicon (Pittsburgh, PA). Anti-CDK4 was from Sigma. Anti-p50 and anti-p65 antibodies for EMSA supershift were from Santa Cruz Biotechnology. LipofectAMINE 2000, Dulbecco's modified Eagle's medium (DMEM), and antibiotic mixture (10,000 units of penicillin, 10,000 mg of streptomycin) were products of Invitrogen. Fetal bovine serum was purchased from Biological Industries (Beit Haemek, Israel).

Cell Culture and Treatments—ML-1, ML-1 PreS1Δ, ML-1 PreS2Δ, ML-1 vector HuH-7 inducible-PreS1Δ, HuH-7 inducible-PreS2Δ, HuH-7 inducible-vector, and MCF-7 cell lines were maintained at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmid and Stable Clone Cell Lines Construction—Plasmid p(3A) SAgΔ1, p(3A) SAgΔ2, and pTK-neo were from Dr. Ih-Jen Su. ML-1 cells were co-transfected with p(3A) SAgΔ1/pTK-neo or p(3A) SAgΔ2/pTK-neo by using Invitrogen LipofectAMINE 2000 reagent according to the manufacturer's protocol. Cells were then selected by G418 for 2 weeks. The p(3A) SAgΔ1 or p(3A) SAgΔ2 stable clone cell lines were established by HBsAg ELISA kit. HuH-7 inducible-PreS1Δ, HuH-7 inducible-PreS2Δ, and HuH-7 inducible-vector cell lines were obtained from Dr. Ih-Jen Su. IκB kinase dominant negative mutant was kindly provided by Dr. Ching-Chow Chen (47).

Pon-A-inducible Expression of the Mutant Type Pre-S—HuH-7-inducible PreS 1Δ, HuH-7-inducible PreS2Δ, and HuH-7-inducible vector cell lines were gifts from Dr. Ih-Jen Su. The Pre-S plasmid constructs contained ponasterone A (pon-A)-controlling elements. HuH-7 cells were co-transfected with these constructs and the vector pERV3, and stable clones were selected by G418 and hygromycin. The stable clones were treated with 10 μM ponasterone A for 0, 24, 48, 72, and 96 h, and then cell lysates of these were extracted for Western blotting.

Preparation of Cytosolic and Nuclear Extracts—ML-1 cells (1×10^6) in 10-cm dishes were incubated for 0, 6, 12, 18, and 24 h in serum DMEM containing 2.5 μg/ml tunicamycin. After treatment, the cells were washed with cold PBS, collected with a cell scraper, harvested by centrifugation, and then by using an NE-PER nuclear and cytoplasmic extraction reagents kit treated to extract their cytosolic and nuclear proteins.

Western Blot Analysis—Cell lysates were prepared by treating cells with 2× SDS lysis buffer (0.1 M Tris (pH 6.8), 0.4% SDS, and 20% glycerol). The protein concentration of the supernatant was measured using a Micro BCA™ protein assay reagent kit. About 15–25 μg of cell lysates were separated by SDS-PAGE with 10% acrylamide and transferred onto polyvinylidene fluoride membranes (Pierce). Following blocking with 5% nonfat dry milk for 1 h at room temperature and washing with Tween 20 with Tris-buffered saline (TTBS), the polyvinylidene fluoride membranes were incubated overnight at 4 °C with primary antibody in TTBS containing 1% bovine serum albumin. The second anti-mouse antibody-horseradish peroxidase conjugate (1:2000 dilution) was subsequently incubated with membranes for 1 h at room temperature and washed extensively for 40–50 min with TTBS at room temperature. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

Immunofluorescence—5×10^5 cells/well were plated in 4-well chambers in DMEM and treated with 2.5 μg/ml tunicamycin for 6 h. Cells for immunofluorescence microscopy of NF-κB were fixed with 3.7% paraformaldehyde for 5 min and washed three times with PBS. Cells were then treated with ice-cold acetone for 1 min and washed three times with PBS. Cells were stained for NF-κB translocation using anti-p65 antibody overnight at 4 °C and then anti-rabbit FITC-conjugated antibody for 1 h. The negative control was cells stained with FITC-conjugated antibody alone. After staining with antibody, cells were viewed with a fluorescence microscope.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides corresponding to the NF-κB consensus sequences in the murine *cox-2* promoter (5′-GAGGTGAGGGGATTCCCTTAGTTAG-3′) were synthesized, annealed, and end-labeled with [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Biosciences) by T4 polynucleotide kinase. Nuclear protein (5 μg) was incubated for 30 min at room temperature with 2 μg of poly(dI-dC) from Amersham Biosciences, 4 μl of gel shift binding 5× buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 50 mM Tris-HCl (pH 7.5), 250 mM NaCl), and 100,000 cpm (1 ng) of a ^{32}P-labeled oligonucleotide in a final volume of 20 μl. Supershift antibodies (2 μg) were added as indicated. DNA-protein (NF-κB) complexes were resolved at 180 V for 4 h in a TBE-buffered, native 5% polyacrylamide gel, dried, and visualized with autoradiography using a Fuji Imaging plate in BAS-IP MS 2025 machine.

Transgenic Mice Tissue Protein Extraction—The transgenic mouse liver, kidney, and muscle tissues were gifts from Dr. Ih-Jen Su. The Pre-S2Δ transgenic mice were constructed by injection of Pre-S2Δ gene fragment into the male pronucleus of fertilized mouse ova. Microinjection was performed in Fvb/n mice. After 15 months, liver, kidney, or muscle tissue from Pre-S2Δ transgenic mice was homogenized in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). Homogenates were centrifuged at 15,000 × *g* for 10 min at 4 °C, and the supernatants were collected. Total protein concentrations of the tissue lysates were quantified using the Micro BCA™ protein

assay reagent kit following the manufacturer's instructions.

RT-PCR—After treatment, the cells were washed with cold PBS and then cells were harvested. Total RNA was extracted from ML-1 cells using VIOGENE (total RNA extraction kit) according to the manufacturer's instruction. The cDNA was reverse-transcribed from 1 μg of total RNA using oligo(dT) primers and Moloney murine leukemia-virus transcriptase. The 5′ and 3′ primers for mouse *cox-2*-specific gene were 5′-ACT CAC TCA GTT TGT TGA GTC ATT-3′ (sense) and 5′-TTT GAT TAG TAC TGT AGG GTT ATT-3′ (antisense). The cycling parameters were as follows: 1 min at 94 °C for denaturation, 1 min at 52 °C for primer annealing, and 1 min at 72 °C for polymerization. Meanwhile, the same amount of cDNA was amplified for 25 cycles using specific glyceraldehyde-3-phosphate dehydrogenase primers: 5′-TGAAGGTCG-GTGTGAACGGATTTGGC-3′ (sense) and 5′-CATGTAGGCCATGAG-GTCCACCAC-3′ (antisense). The products were visualized after electrophoresis on a 1.5% agarose gel containing ethidium bromide. The signal level of the bands was quantified densitometrically.

RT-PCR for Human HBsAg Type II Hepatoma—The HBsAg type II cells were obtained by micro-laser dissection, and total RNA was extracted for RT-PCR. The cDNA was reverse-transcribed from 1 μg of total RNA using oligo(dT) primers and Moloney murine leukemia-virus transcriptase. The 5′ and 3′ primers for the human *COX-2*-specific gene were 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ (sense) and 5′-AGA TCA TCT GTG CCT GAG TAT CTT-3′ (antisense). The cycling parameters are as follows: 1 min at 94 °C for denaturation, 1 min at 52 °C for primer annealing, and 1 min at 72 °C for polymerization. Meanwhile, the same amount of cDNA was amplified for 25 cycles by using specific β-actin primers: 5′-ATC ATG TTT GAG ACC TTC AA-3′ (sense) and 5′-CAT CTC TTG CTC GAA GTC CA-3′ (antisense). The products were visualized after electrophoresis on a 1.5% agarose gel containing ethidium bromide. The signal level of the bands was quantified densitometrically.

PGE_2 EIA Immunoassay—5×10^5 cells/well were plated in 6-well dishes in DMEM and cultured for 24 h. PGE_2 levels in the supernatant conditioned medium were then assayed for using a prostaglandin (PG) E_2 EIA kit.

HBsAg ELISA Kit—Cells were washed with cold PBS and then collected with a cell scraper and harvested by centrifugation. The supernatant was removed; 100 μl of H_2O was added, and then the cells were freeze-thawed three times at −80 °C. The level of HBsAg was determined using an ELISA kit following the manufacturer's instructions.

Statistical Analysis—Results were presented as the mean ± S.D., and statistical comparisons were made using the Student's *t* test. Significance was defined at the $p < 0.05$ or 0.01 level.

RESULTS

ER Stress Induced the Expression of COX-2—Cells of the mouse liver immortalized cell line ML-1 were treated with 2.5 μM TM, and the expression of COX-2 was determined by Western blotting and RT-PCR. COX-2 mRNA started to increase 3 h after tunicamycin treatment (Fig. 1*A*), and COX-2 protein was induced 6–12 h after treatment with either tunicamycin or another ER stress inducer brefeldin A. The expression of GRP78, an unfolded protein response chaperone, was enhanced in response to tunicamycin (Fig. 1*B*). Similarly, the expression of COX-2 protein was enhanced by tunicamycin in MCF-7 human breast cancer cells (Fig. 1*C*).

COX-2 Induction Was Transcription-dependent and Nuclear Export-dependent—To elucidate the mechanism of COX-2 induction, ML-1 cells were treated with tunicamycin with or without the transcriptional inhibitor actinomycin D. Actinomycin D alone did not alter COX-2 and did abolish tunicamycin-induced COX-2 induction (Fig. 2*A*). The expression of COX-2 may be regulated at the nuclear export level (48); therefore, we studied whether the induction of COX-2 by tunicamycin can be inhibited by leptomycin B (LMB), a nuclear export inhibitor. LMB inhibited the COX-2 induction in a dose-dependent fashion within the range 2.5 to 10 μM (data not shown). At the concentration of 5 μM, LMB can inhibit COX-2 induction more than 90% throughout the 24-h time course (Fig. 2*B*). On the other hand, the induction of GRP78 was inhibited by actinomycin D but not leptomycin.

COX-2 Induction Is NF-κB-dependent—Transcription factor

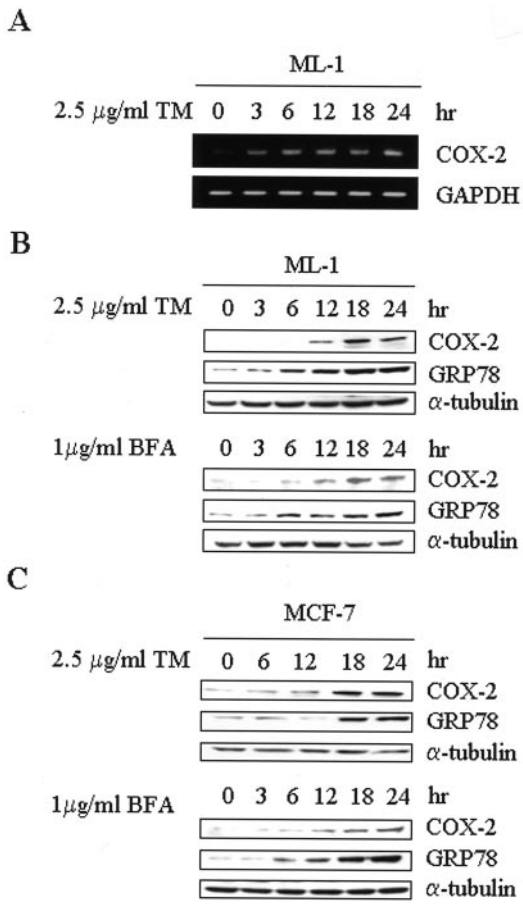

FIG. 1. **Elevated expression of COX-2 in response to endoplasmic reticulum stress.** *A*, ML-1 cells were treated with tunicamycin for 0, 3, 6, 12, 18, and 24 h. Total RNA was isolated and then subjected to RT-PCR analysis. ML-1 (*B*) and MCF-7 cells (*C*) were treated with tunicamycin (*TM*) or brefeldin A (*BFA*) for various times, and cell lysates were analyzed by Western blotting with antibodies for COX-2, GRP78, and α-tubulin. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

NF-κB can regulate the expression of COX-2 and may be induced by ER stress. ML-1 hepatocytes were treated with tunicamycin for the indicated time, and the amount of nuclear translocation of NF-κB was determined by Western blot analysis of the nuclear and cytosolic fraction. Increased nuclear translocation of the p50 and p65 subunits of NF-κB was observed after tunicamycin treatment in ML-1 cells with different kinetics (Fig. 3*A*). Degradation of cytosolic IκBα during ER stress may explain the nuclear translocation of NF-κB (Fig. 3*A*). Nuclear translocation of the p65 subunit of NF-κB was further confirmed by immunofluorescence in ML-1 cells and MCF-7 cells (Fig. 3*B*). Two forms of NF-κB complexes were detected with gel shift analysis, and the upper gel-shifted band of NF-κB DNA binding activity was strongly induced at 1.5 and 3 h after ER stress (Fig. 3*C*). Anti-p65 antibody supershifted the upper gel-shifted band but did not affect the lower gel-shifted band. In contrast, anti-p50 antibody supershifted the lower gel-shifted band completely, and partly decreased the intensity of the upper gel-shifted band. Control anti-c-Jun antibody did not affect the NF-κB DNA binding activity. Therefore, the lower gel-shifted band may consist of p50/p50 homodimer only, and the upper gel-shifted band may contain both p65/p65 homodimer and p65/p50 heterodimer. Activation of NF-κB appears to be mediated by the increase of p65/p65 and p65/p50 DNA binding activity (Fig. 3*C*). To determine whether NF-κB is essential for the induction of COX-2, ML-1 cells were

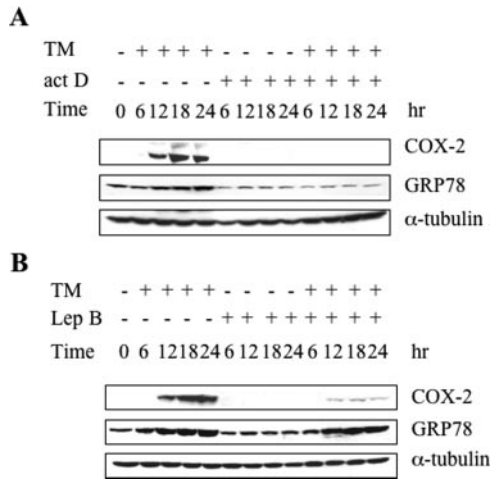

FIG. 2. **Transcription and nuclear export are required for COX-2 induction.** ML-1 cells were incubated with tunicamycin and/or transcriptional inhibitor actinomycin D (*act D*) (*A*) and the nuclear export inhibitor leptomycin B (*Lep B*) (*B*). The expressions of COX-2 and GRP78 were analyzed by Western blotting. α-Tubulin is an internal control.

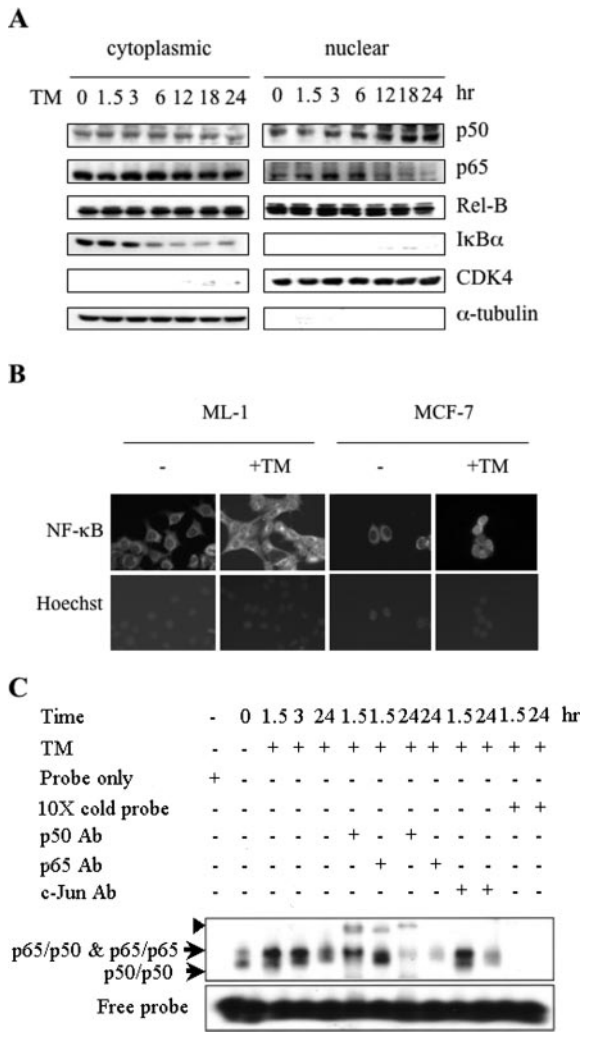

FIG. 3. **Activation of NF-κB during ER stress.** *A*, ML-1 cells were incubated with tunicamycin for 0, 1.5, 3, 6, 12, 18, and 24 h, and NF-κB subunits in the cytoplasmic and nuclear fractions were analyzed by Western blotting with antibodies against subunits p65, p50, Rel-B, and IκBα. Cyclin-dependent kinase 4 (*CDK4*) and α-tubulin were used as internal markers for nuclear and cytoplasmic proteins. *B*, ML-1 cells were treated with tunicamycin for 6 h and probed with anti-p65 antibody and FITC-conjugated secondary antibody. Hoechst staining revealed the nucleus. *C*, nuclear NF-κB DNA binding activity was measured by EMSA using a probe corresponding to the NF-κB-binding site of the murine *cox-2* promoter. The *arrows* indicated the two NF-κB complexes, and the *arrowhead* indicates the supershifted complexes with anti-p65 subunit or anti-p50 subunit antibody (*Ab*). Anti-c-Jun antibody serves as a control for supershift control.

treated with the NF-κB inhibitor PDTC. PDTC blocked the tunicamycin induction of COX-2 expression in a dose-dependent fashion (Fig. 4*A*). The time course for inhibition of COX-2 induction was measured in the presence of 50 μM PDTC (Fig. 4*A*). Another NF-κB inhibitor Bay 11-7082, an IκB kinase inhibitor, inhibited the COX-2 induction in a similar fashion. The degradation of IκBα was attenuated by Bay 11-7082 (Fig. 4*B*). Furthermore, the induction of COX-2 was inhibited by the expression of the dominant negative mutant of IκB kinase in ML-1 cells (Fig. 4*C*). PDTC, Bay 11-7082, or IκB kinase dominant negative mutant did not alter the expression of GRP78, suggesting that NF-κB is not involved in that branch of UPR pathway. These results altogether indicated that ER stress induced COX-2 in an NF-κB-dependent pathway.

p38 MAPK Is Required for COX-2 Induction—As the expression of COX-2 is regulated through MAP kinases too, we investigated whether the MAPKs played a role in the induction of COX-2. ML-1 cells were treated with 2.5 μg/ml tunicamycin, and the phosphorylated active form or total extracellular signal-regulated kinase (ERK), p38, and JNK were examined with Western blotting. All three MAPKs were activated but with different kinetic fashion (Fig. 5*A*). Phosphorylated ERK (PERK) appeared at 2 h and phosphorylated JNK (pJNK) appeared at 6 h after tunicamycin treatment. On the other hand, phosphorylated pp38 occurred at an early time (30 min to 3 h) and stayed for 24 h (Fig. 5*A*). The requirement of various forms of MAPK was determined with the addition of 1–10 μM MAPK inhibitors, SB203580, PD98059, and JNK inhibitor II, respectively, and COX-2 levels were determined from Western blots. p38 MAPK inhibitor SB203580 as low as 1 μM can inhibit the induction of COX-2 (Fig. 5*B*). One μM ERK inhibitor PD98059 had no effect, and higher doses (5 and 10 μM) did inhibit the induction (Fig. 5*C*). In contrast, JNK was not involved in the induction of COX-2 as the JNK inhibitor had no effect (Fig. 5*D*).

Induction of NF-κB Is Dependent on pp38 MAPK and Phosphorylation of eIF2α—Phosphorylation of the α subunit of eIF2α is required for the activation of NF-κB in response to diverse stresses (31); therefore, we examined whether inhibition of eIF2α-phosphorylation by 2-aminopurine (2-AP) affects the expression of COX-2. 2-AP alone did not alter the expression of COX-2 or GRP78 (data not shown). Both phosphorylation of eIF2α and the expression of COX-2 was attenuated by 10 mM 2-AP (Fig. 6*A*). 2-AP delayed and inhibited the nuclear translocation of p65 and p50 subunits of NF-κB (Fig. 6*B*). EMSA analysis further confirmed significant inhibition of NF-κB DNA binding activity by 2-AP at 1.5 and 3 h after ER stress (Fig. 6*C*). On the other hand, pp38 kinase inhibitor SB203580 only mildly delayed the nuclear translocation of p50 and p65 subunits of NF-κB but significantly inhibited NF-κB DNA binding activity at 1.5 and 3 h after ER stress (Fig. 6, *B* and *C*). The JNK inhibitor had no effect on either COX-2 expression or NF-κB DNA binding activity (Figs. 5*D* and 6*C*). Therefore, activation of NF-κB DNA binding activity is mediated through eIF2α and pp38 MAPK.

Hepatitis B Virus Mutant Large Surface Protein Can Induce COX-2 in Vitro—ER stress can be induced by either drugs such as tunicamycin or by overexpression of mutant proteins including virus gene products. In hepatitis B virus-associated hepatocarcinoma, deletions in the Pre-S1 or Pre-S2 region of hepatitis B virus large surface proteins have been detected (49). These hepatitis B virus mutant large surface proteins were

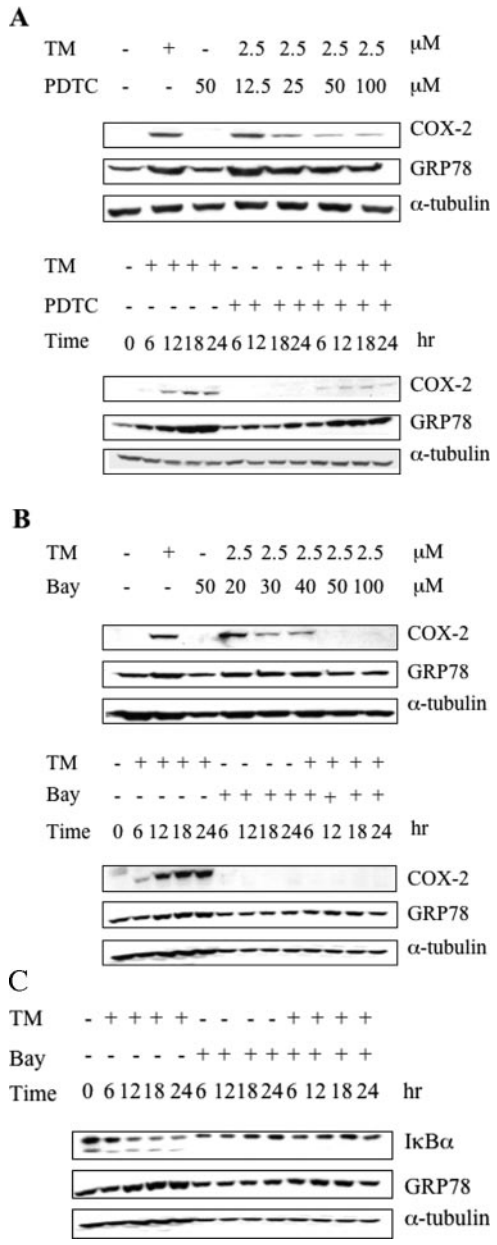

FIG. 4. **Attenuation of COX-2 expression by NF-κB inhibitors and IκB kinase dominant negative mutant.** *A*, ML-1 cells were treated with tunicamycin and increasing doses of PDTC or 50 μM PDTC for various times. *B*, ML-1 cells were treated with tunicamycin and increasing doses of Bay 11-7082 or 50 μM Bay 11-7082 for various times, and the expressions of COX-2, IκBα, and GRP78 were analyzed by Western blotting. *C*, ML-1 cells were transfected with IκB kinase dominant negative mutant IKK2M or pCDNA3.1 vector control, and the expressions of COX-2 and GRP78 were measured.

shown to induce ER stress (11, 12). Therefore, it is important to investigate whether the mutant large surface protein can induce the expression of COX-2. The expressions of COX-2 and GRP78 were examined in the following cell lines: ML-1 cells, ML-1 transfectants expressing two types of Pre-S mutant surface proteins (49), and an ML-1 control transfectant. The expression of COX-2 was elevated in ML-1 cells expressing hepatitis mutant surface proteins but not in control transfectants (Fig. 7*A*). One of the major products of COX-2, prostaglandin E_2, was measured in ML-1 cells expressing Pre-S mutant surface proteins. Prostaglandin was elevated 4–5-fold by the expression of Pre-S mutant surface proteins (Fig. 7*B*). That the expression of COX-2 induced by hepatitis B mutant large surface proteins requires the activation of pp38 MAPK and tran-

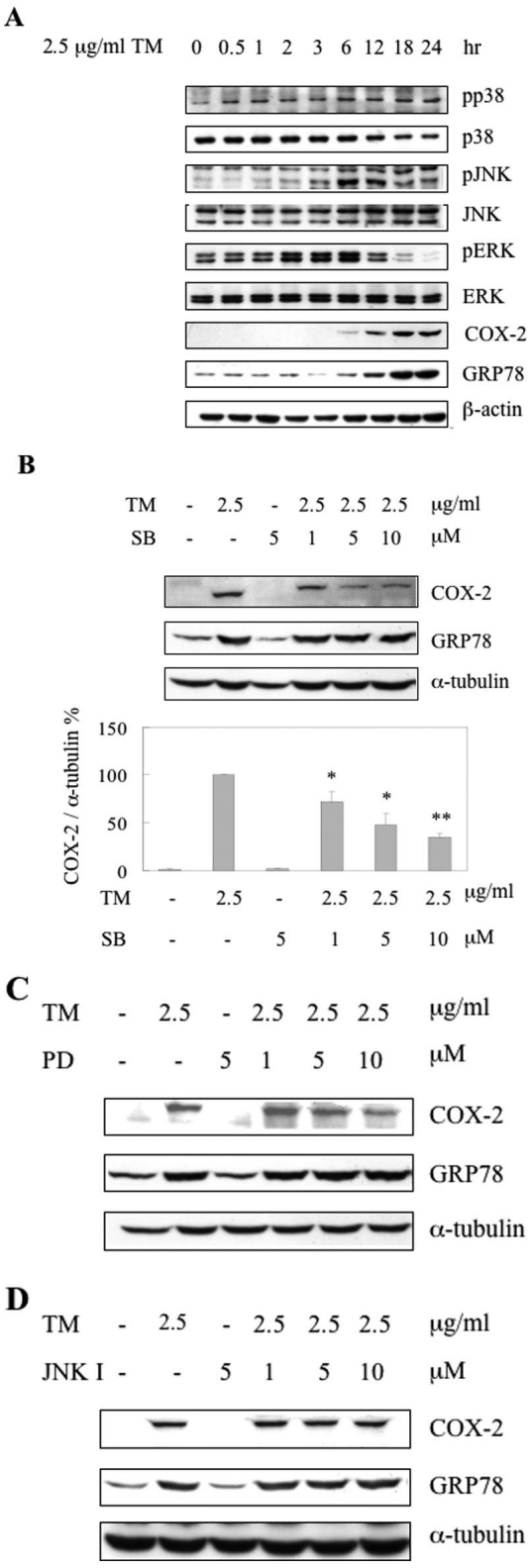

FIG. 5. **Activation of p38 MAPK is essential for COX-2 induction during ER stress.** *A*, ML-1 cells were treated with TM for the times indicated, and the activation of MAPK pathways was determined by Western blot using antibodies specific for active forms of MAPK or total MAPK. COX-2, GRP78, and α-tubulin were indicators for endoplasmic reticulum stress and internal control. *B–D*, ML-1 cells were treated with tunicamycin as follows: *B*, pp38 inhibitor SB203580 (*SB*); *C*, ERK inhibitor PD98059 (*PD*); *D*, JNK inhibitor, and the expression of COX-2 and GRP78 was determined with Western blotting analysis. The amount of COX-2 induction and attenuation by pp38 inhibitor SB203580 was semi-quantitated by densitometry scanning in three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

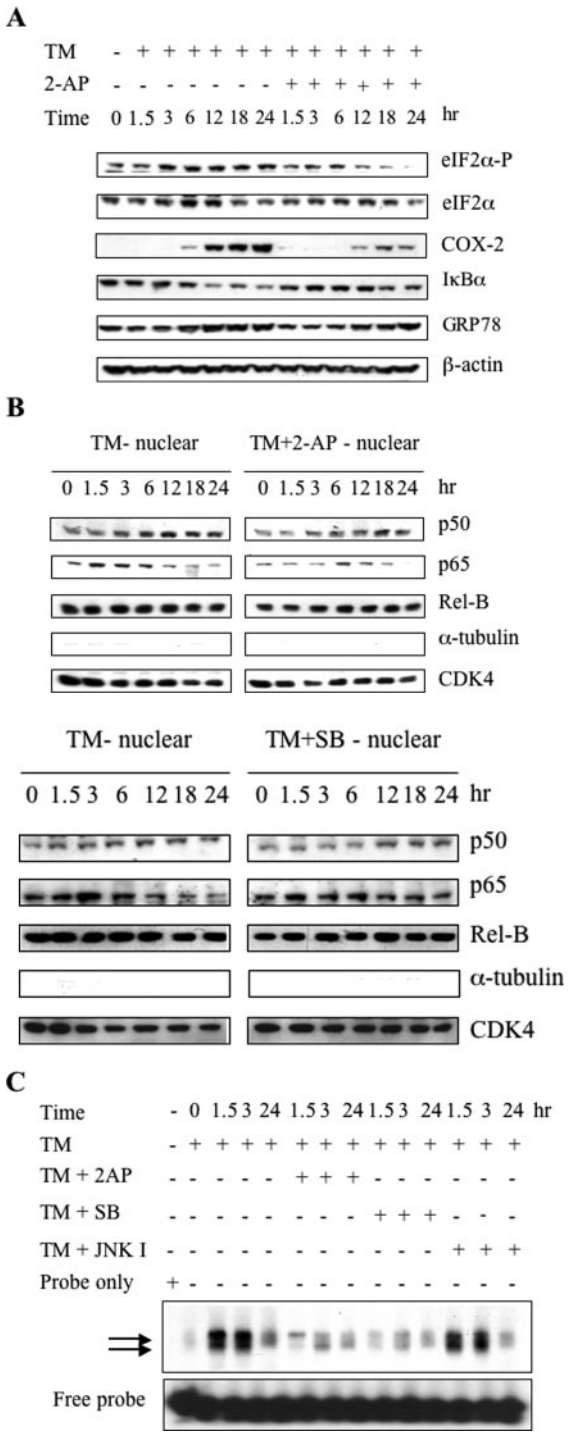

FIG. 6. **Activation of NF-κB is mediated through α subunit of eIF2α phosphorylation and p38 MAPK.** ML-1 cells were treated with TM and eIF2α phosphorylation inhibitor 2-AP or pp38 inhibitor SB203580 for the indicated times. *A*, the expression of phosphorylated form of eIF2α, total eIF2α, COX-2, IκBα, and GRP78 in total cell lysates was determined with Western blotting. *B*, NF-κB complex subunits in the nuclear fractions were analyzed by Western blotting with antibodies against subunits p65, p50, Rel-B, and IκBα. Cyclin-dependent kinase 4 (*CDK4*) and α-tubulin were used as internal markers for nuclear and cytoplasmic proteins respectively. *C*, nuclear NF-κB DNA binding activity was measured with EMSA using a probe corresponding to the NF-κB-binding site of the murine *cox-2* promoter. The *arrows* indicated the two NF-κB complexes. JNK inhibitor did not affect NF-κB DNA binding activity, serving as a control.

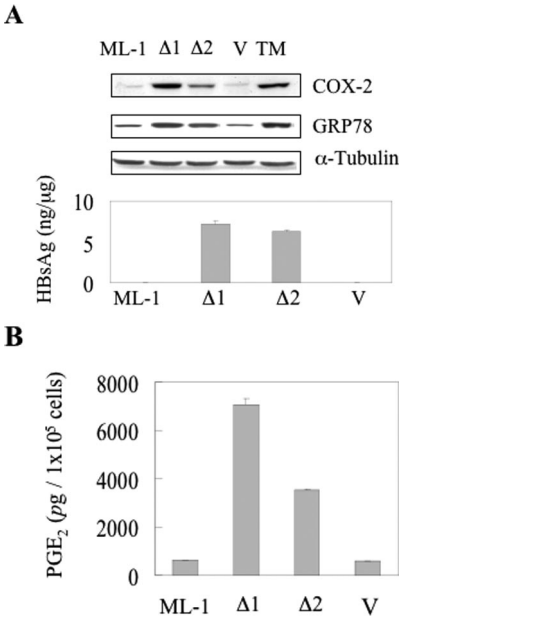

FIG. 7. **Increase of GRP78, COX-2 protein, and PGE_2 in ML-1 cells expressing del-PreS1 (Pre-S1Δ) and del-PreS2 (Pre-S2Δ) mutant HBV large surface protein.** *A*, the *upper panel*, Western blot analysis of COX-2 protein and GRP78 expression in *1st lane*, ML-1; *2nd lane*, ML-1-Δ1 (*Δ1*); *3rd lane*, ML-1-Δ2 (*Δ2*); *4th lane*, ML-1-neo (*V*), and *5th lane*, ML-1 cells treated with tunicamycin; the *lower panel* shows HBV large surface proteins expression as determined by HBsAg ELISA. *B*, the level of COX-2 product PGE_2 in culture medium was determined by EIA.

scription factor NF-κB was demonstrated by the inhibition of COX-2 expression by p38 inhibitor SB203580 and NF-κB inhibitor Bay 11-7082 (Fig. 8*A*). The production of PGE_2 was also significantly attenuated (Fig. 8*B*). To demonstrate further the role of the HBV mutant large surface proteins in the induction of COX-2 *in vitro*, mutant HBV large surface proteins were expressed in the presence of pon-A, an inducible promoter in HuH-7 cell lines. Stable transfectants and control transfectants were induced by the addition of pon-A, and the expressions of COX-2 and GRP78 were measured (Fig. 9*A*). Inducible expression of mutant large surface proteins was quantitated by EIA against HBsAg (Fig. 9*B*). Mutant HBV large surface proteins can enhance the expression of COX-2 and GRP78.

Hepatitis B Virus Mutant Large Surface Protein Can Induce COX-2 in Vivo—To confirm further that deletion forms of mutant HBV large surface proteins can induce COX-2 *in vivo*, we created transgenic mice that express the Pre-S2 deletion form of HBV large surface protein under the control of its native promoter. The expression of HBV large surface protein was detected in the liver and kidney (Fig. 9). Elevated expression of COX-2 was observed in liver and kidney tissue in male or female mice (Fig. 10). In addition, we used laser capture dissection to isolate type II hepatoma cells expressing the Pre-S2 deletion form of HBV large surface proteins from human tumor tissue (48), and RT-PCR to quantify the COX-2 mRNA. High expression of COX-2 mRNA was observed in hepatocytes that express the deletion forms of HBV large surface proteins (Fig. 10*B*). These results altogether demonstrate that Pre-S mutant surface proteins can induce COX-2, and the induction is possibly mediated through ER stress, at least partly.

Hepatitis B Virus Mutant Large Surface Proteins Enhance Anchorage-independent Growth in Soft Agar—The expression of COX-2 was associated carcinogenesis; therefore, the expression of Pre-S mutant HBV large surface proteins may enhance cellular transformation through the generation of COX-2. The expression of Pre-S mutant surface proteins containing deletions in either the Pre-S1 region or Pre-S2 region enhanced the growth in soft agar (Fig. 11*A*), which is an indication for anchorage-independent growth. Addition of COX-2 inhibitor

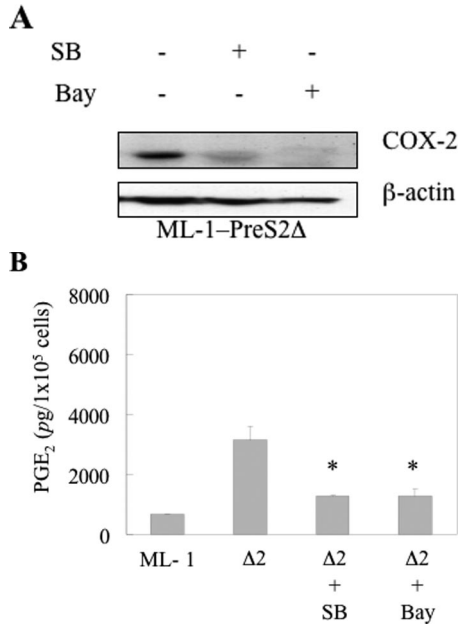

FIG. 8. **Attenuation of COX-2 expression and PGE_2 production by p38 MAPK inhibitor (SB203580) and NF-κB inhibitor (Bay 11-7082).** *A*, ML-1 cells expressing PreS2Δ were incubated with SB203580 (*SB*) or Bay 11-7082 (*Bay*) for 24 h. The expression of COX-2 was determined by Western blotting. *B*, the PGE_2 in culture medium was determined by EIA. *, $p < 0.05$.

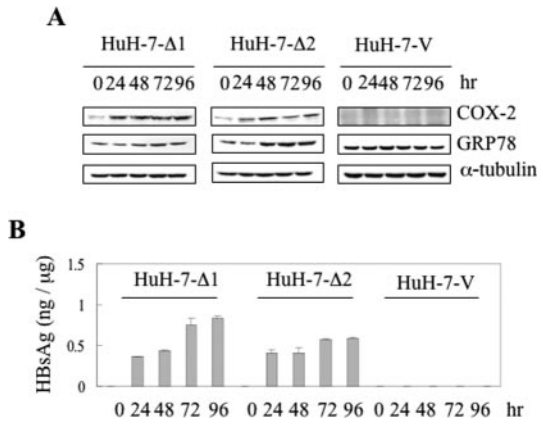

FIG. 9. **Inducible expression of hepatitis B large surface proteins enhanced the expression of COX-2 in HuH-7 cells.** PreS1Δ and PreS2Δ HBV large surface proteins were induced by addition of ponasterone A, and the expression of COX-2 and GRP78 (*A*) and HBV large surface proteins (*B*) was determined by Western blotting and EIA, respectively.

etodolac to ML-1 cells expressing mutant surface proteins significantly inhibited their growth on soft agar (Fig. 11*A*). Inducible expression of mutant large surface proteins in HuH-7 cells also enhanced growth in soft agar, and the addition of etodolac completely abolished this growth (Fig. 11*B*).

DISCUSSION

ER stress can induce multiple signal pathways including the ATF6/IRE1 UPR pathway, eIF2α pathway, and NF-κB EOR pathway (19–28). These pathways may cross-talk with each other or converge on common downstream effectors (50). In this report, we have demonstrated that ER stress can induce the expression of COX-2, and the induction is dependent on the transcription factors NF-κB and p38 MAPK. The enhancement of NF-κB DNA binding activity also requires p38 MAPK activation and eIF2α phosphorylation (Fig. 12). IRE1 or eIF2α is involved in activation of transcription factor NF-κB during

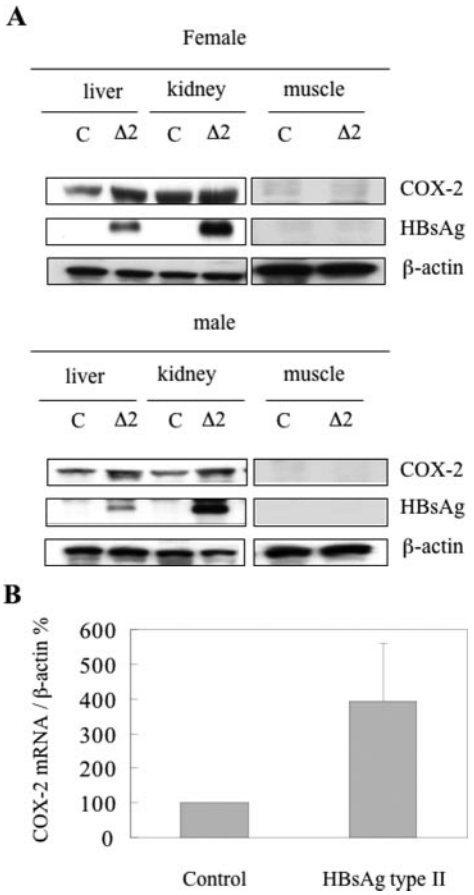

FIG. 10. **Elevated COX-2 is associated with PreS2Δ expression *in vivo*.** *A*, COX-2 expression was elevated in transgenic mice expressing PreS2 deletion (Δ2) HBV large surface protein. The expression of COX-2 in liver, kidney, and muscle tissues (*C*, control; Δ2, PreS2Δ) was determined by Western blotting. *B*, COX-2 mRNA expression was elevated in human HBsAg type II hepatoma cells expressing mutant HBV large surface protein. The normal and HBsAg type II hepatocytes were isolated by laser capture microdissection from hepatoma tissue. Total RNA was isolated, and RT-PCR was used to measure COX-2 and β-actin.

endoplasmic reticulum stress (30, 31). Phosphorylation of eIF2α is also essential for activation of NF-κB in our experimental system. NF-κB and p38 MAPK may not be the upstream signal for the ATF6 pathway, because the induction of GRP78 by ER stress was not affected by the inhibitors for p38 MAPK and NF-κB. Recently, pp38 activation was reported to be mediated through IRE1 because ER stress-induced pp38 activation was attenuated in IRE1-deficient cells (51). Therefore, IRE1-dependent activation of NF-κB may also partly mediated through the p38 MAPK pathway (Fig. 12). Endoplasmic reticulum stress can be induced by *N*-glycosylation-inhibition through tunicamycin or by expression of mutant viral surface proteins such as hepatitis B virus large surface proteins. The induction of COX-2 by drugs or overexpression of mutant proteins was similarly mediated through p38 MAPK and NF-κB. However, the details of regulation may not be similar (*i.e.* the pathway to NF-κB activation may be different in tunicamycin-treated ML-1 cells and ML-1 cells expressing mutant HBV surface proteins). Preliminary data suggest that calcium ion is required for tunicamycin-induced NF-κB but is not essential for the induction of NF-κB in cells expressing HBV large surface proteins (data not shown).

Nuclear translocation of NF-κB subunit p65 occurred ~1.5 h after ER stress, and the p50 subunit translocated later (Fig. 3*A*). NF-κB DNA binding activity increased significantly at

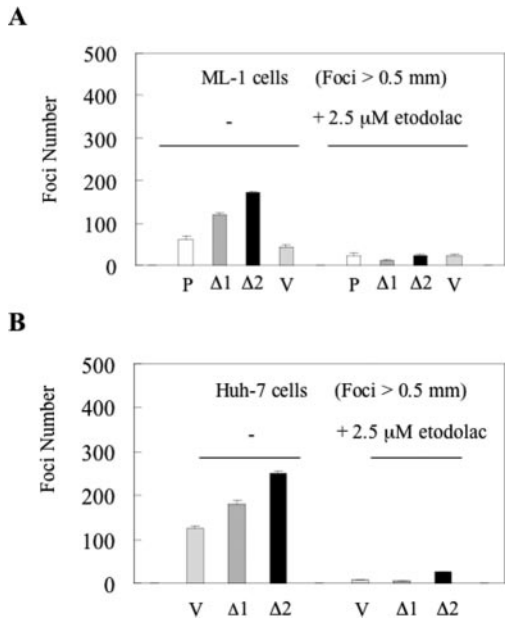

FIG. 11. **Expression of PreS1Δ and PreS2Δ HBV large surface protein enhanced anchorage-independent growth, which was abolished by COX-2 inhibitor.** PreS1Δ (Δ*1*) or PreS2Δ (Δ*2*) HBV large surface proteins were stably expressed in ML-1 cells (*A*) or inducibly expressed in HuH-7 cells (*B*). Ten thousand cells were cultured in 6-well plates containing 0.35% soft agar, and the foci (>0.5 mm) number was determined 3 weeks later with or without 2.5 mM COX-2 inhibitor etodolac. Inducer ecodynosine was added in the medium of HuH-7 transfectants for the induction of HBV large surface proteins. *P*, parental cell; *V*, vector control.

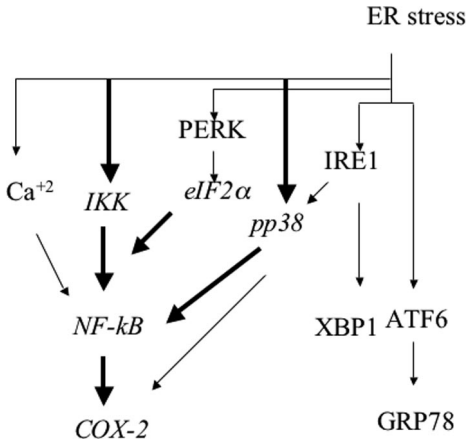

FIG. 12. **Signaling of endoplasmic reticulum stress to COX-2.** Accumulation of unfolded or malfolded proteins in the endoplasmic reticulum leads to activation of multiple signaling pathways. First, activation of IRE1 and ATF6 represents the standard UPR pathways and turns on the expression of many downstream genes including *GRP78*. Second, activation of ER localized kinase (*PERK*) to decrease the translation rate is mediated through a change in eIF2α phosphorylation. Third, activation of p38 MAPK and NF-κB may alter cellular homeostasis by regulating multiple genes including *cox-2*. This pathway (indicated by *heavy arrows*) is demonstrated in this report. These three pathways are not independent from each other; for example, activation of NF-κB is dependent on phosphorylation of eIF2α subunit and activation of p38 MAPK.

early times during ER stress and was mainly composed of p65/p65 and p65/p50 complexes (Fig. 3*C*). Degradation of IκB was observed 3–6 h after ER stress, which is a little later than the nuclear translocation of NF-κB. This phenomenon may be because of the possibility that the phosphorylation of IκB and release of p65 subunit precedes the degradation of IκB. Alternatively, other non-IKK kinases may be partly involve in phosphorylation of IκB and release of p65 (52). In our ER stress model, activation of p38 MAPK may be involved in enhancing nuclear NF-κB DNA binding activity because inhibition of p38 MAPK decreases the DNA binding activity of NF-κB with minor effects on nuclear translocation of NF-κB subunits. Recently, p38 MAPK was shown to activate the NF-κB transcriptional activity without affecting its nuclear translocation (53, 54), which may be similar to the role of p38 MAPK in this report. The appearance of NF-κB in the nucleus began 1.5 h after ER stress which is consistent with the induction of COX-2 mRNA. COX-2 protein expression increased significantly 12–18 h after ER stress. The NF-κB appears to be essential for the induction of COX-2 mRNA; however, NF-κB alone may not be sufficient to fully induce the expression of COX-2 protein. P38 MAPK may not only act on transcriptional level of COX-2 mRNA but may also regulate the stability of COX-2 mRNA (55, 56). In addition, activation of p38 MAPK may further enhance the activation of the NF-κB-dependent gene (57). Therefore, coordination of NF-κB and p38 MAPK may be required for the full induction of COX-2 protein.

Alterations in endoplasmic reticulum homeostasis trigger a complex series of events, including synthesis of chaperone, decrease of translation, and degradation of unfolded proteins to promote cellular survival. Endoplasmic reticulum stress induced p53 cytoplasmic localization and prevented p53-dependent apoptosis (58). Endoplasmic reticulum stress induced by glucose depletion could enhance the expression of phospho-glycoprotein (59), which may affect cancer outcome. Breast cancer cells can secrete pro-angiogenic vascular endothelial growth factor in response to ER stress (60). In this report, we demonstrated the following: 1) the expression of COX-2 may be induced by expression of surface proteins in cells with latent virus infection or other stimuli, including drugs that perturb ER function, and 2) the COX-2 pathway may be used to enhance anchorage-independent growth. This further extends the impact of endoplasmic reticulum stress on cellular carcinogenesis.

Drug-induced ER stress leads to degradation of IκB and to nuclear translocation of NF-κB in hepatocytes and breast cancer cells. Degradation of IκB was not observed in the ER stress-induced activation of NF-κB in mouse embryo fibroblasts (31). This minor discrepancy may be cell type-specific. Similarly, ER stress-induced GSK3β activation plays an opposite role in different cell types (61). The importance of NF-κB in the induction of COX-2 has been demonstrated in many reports (41–44, 62, 63). Furthermore, NF-κB plays an important role in liver carcinogenesis (64, 65). Activation of NF-κB was observed frequently in hepatocarcinoma, and the essential role of NF-κB for cancer growth was confirmed in several human cancer cell lines (66, 67). Therefore, control of NF-κB activity may be an important therapeutic target for the treatment of human hepatocarcinoma (68).

Because latent hepatitis B and hepatitis C virus infection is clearly associated with hepatocarcinogenesis, and the expression of viral surface proteins induces endoplasmic reticulum stress and COX-2 expression, COX-2 is therefore a rational chemopreventive target for hepatocarcinoma. COX-2 inhibitor is a chemoprevention agent for colon cancer, and its use has been proposed recently (69, 70) to decrease the incidence of other types of cancer including gastric and lung cancer. COX-2 inhibitor is used to treat hepatoma (71, 72), but its role in prevention is not clearly defined (73). Recently a COX-2 inhibitor was shown to prevent hepatocarcinogenesis in an animal model (74). Our results strongly suggest that preclinical investigation of the effect of COX-2 inhibitors on HBV carrier status is warranted.

Induction of endoplasmic reticulum stress in carcinogenesis may be mediated through latent expression of viral surface

proteins or by nutrient deprivation. These latent endoplasmic reticulum stresses induce many survival pathways, including sequestering of p53 to inactivate p53-dependent pathway, expression of the pro-angiogenic factor (vascular endothelial growth factor), and COX-2 (shown in this report). Our study indicated that sites of ER stress should be considered important targets of future carcinogenesis investigations.

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