Endoplasmic Reticulum Stress Response as a Possible Mechanism of Cyclooxygenase-2-independent Anticancer Effect of Celecoxib

WONJAE CHA[1,3], SEOK-WOO PARK[2], TACK-KYUN KWON[1], J. HUN HAH[1,2] and MYUNG-WHUN SUNG[1,2]

[1]*Department of Otorhinolaryngology–Head and Neck Surgery, Seoul National University Hospital, Seoul, Republic of Korea;*
[2]*Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea;*
[3]*Department of Otorhinolaryngology-Head and Neck Surgery and Biomedical Research Institute, Pusan National University, Pusan, Republic of Korea*

Abstract. *Background: We investigated whether the endoplasmic reticulum (ER) stress response could be a cyclooxygenase-2 (COX2)-independent mechanism of growth inhibition by celecoxib in a head and neck squamous cell carcinoma (HNSCC) cell line. Materials and Methods: We performed western blotting and reverse transcription polymerase chain reaction to analyze the expression of ER stress response-associated proteins C/EBP homologous protein (CHOP), glucose-regulated protein (GRP)-78 and X-box binding protein-1 (XBP1), after treatment of celecoxib in the SNU-1041 cell line. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the change in growth inhibition by celecoxib after inhibition of the ER stress pathway by CHOP small-interfering RNA (siRNA). Results: Celecoxib triggered an ER stress response in this HNSCC cell line as shown by activation of CHOP, GRP78 and XBP1. The inhibition of cell proliferation by celecoxib was effectively hindered with CHOP siRNA. Conclusion: ER stress response could be a COX2-independent anticancer mechanism of celecoxib.*

Non-steroidal anti-inflammatory drugs (NSAIDs) play promising roles in the treatment and prevention of various types of cancer (1). Among them, celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is one of the most widely-studied agents. Although celecoxib has shown potent anticancer activity in various animal tumor models (2-5), the underlying molecular mechanisms of anticancer effect are not completely understood. In previous studies with head and neck squamous cell carcinoma (HNSCC) cell lines, COX2-inhibitory chemicals showed tumor-killing effects even in HNSCC cell lines without expression of COX2. Among various COX2 inhibitors, celecoxib has shown outstanding COX2-independent tumor-killing actions (6, 7).

Treatment of cultured cells with various NSAIDs, including celecoxib, increased levels of intracellular calcium, with subsequent activation of the endoplasmic reticulum (ER) stress response (8-11). The primary purpose of the ER stress response is to alleviate the stressful disturbance and restore proper ER homeostasis. However, against intense or persistent ER stress, these pathways will trigger programmed cell death or apoptosis (12). Although these observations suggest that components of the ER stress response might participate in NSAID-induced apoptosis, ER stress has not yet been studied as a COX2-independent mechanism of celecoxib.

The present study investigated whether the ER stress response could be a COX2-independent mechanism of celecoxib action in an HNSCC cell line.

Correspondence to: J. Hun Hah, MD, Ph.D., Seoul National University Hospital, 101 Daehakro, Jongno-gu, Seoul 110-744, Korea. Tel: +82 220720215, Fax: +82 27452387, e-mail: jhunhah@snu.ac.kr

Key Words: Celecoxib, head and neck cancer, endoplasmic reticulum stress response, COX2.

Materials and Methods

Cell culture. SNU-1041 cell line was obtained from the Korea Cell Line Bank (Seoul National University, Seoul, Korea). This single cell line was selected because it is one of the HNSCC cell lines found to have high expression of COX2 in our previous study (13). Unless otherwise stated, all cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA).

Cell proliferation assay. The cells were seeded in 96-well plates and incubated for 24 h at 37˚C and treated with celecoxib at the concentrations of 0-40 μM for 48 hours at 37˚C. After drug treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Saint Louis, MO, USA) assay was performed.

Semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). SNU-1041 cells were treated with 20-30 μM of celecoxib

for 9 h. And then, to extract RNA, cells were treated with 500 μl TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). The lysates were added to 100 μl chloroform and vortexed. After centrifugation at 12,000× *g* and 4˚C for 15 min, the upper colorless layer was separated, precipitated with 100 μl isopropyl alcohol (at 4˚C for 20 min), and centrifuged at 12,000× *g* and 4˚C for 10 min. Pellets were washed with 75% ethanol and centrifuged twice at 7,500 g and 4˚C for 5 min, and the pellets obtained were air-dried and dissolved in nuclease-free water. Complementary DNA (cDNA) was made from 1 μg total RNA, dissolved in 20 μl of the cDNA synthesis kit reaction mixture containing deoxynucleotide triphosphate mixture, 0.5 μg oligo primer, and 15 U avian myoblastosis virus reverse transcriptase (Promega, Madison, WI, USA). This was reacted at 42˚C for 60 min, at 95˚C for 5 min, and then at 4˚C for 5 min. PCR products for C/EBP homologous protein (*CHOP*) mRNA, glucose-regulated protein 78 (*GRP78*) mRNA and X-box binding protein 1 (*XBP1*) mRNA were obtained from 1 μl cDNA with a PCR reagent kit (Genenmed, Seoul, Korea) and a pair of primers 10 pM, which were incubated at 95˚C for 2 min, then reacted for different numbers of cycles at 94˚C (20 s), 64˚C (20 s), and 72˚C (40 s), and incubated at 75˚C for 5 min and at 4˚C for 5 min. Each sample was treated with different dosages of CD and at different levels of acidity (pH). The samples were analyzed on 1% agarose gel after 25 to 35 PCR cycles.

Protein extraction and western blotting. For CHOP detection, SNU-1041 cells were prepared by adding protein lysis buffer (150 mmol/l NaCl, 100 mmol/l Tris, 1% Tween 20, 50 mmol/I diethyl dithiocarbamate, 1 mmol/l EDTA, and 1 mmol/l phenylmethyl-sulfonyl fluoride) and, 24 h later, were treated with CD. After rotation for 40 minutes at 4˚C and centrifugation at 10,000× *g* for 10 min, the supernatants containing the proteins were separated and protein concentrations were measured. Electrophoresis was carried out on 30 μg of protein loaded into 12% sodium dodecyl sulfate-polyacrylamide gel. Resolved proteins were transferred into nitrocellulose membranes for 2 hours. The nitrocellulose sheets were incubated with antibody to CHOP/GADD153 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated with the corresponding secondary antibody (1:1,000 dilutions) conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA). CHOP protein was visualized by developing with enhanced chemiluminescence reagent and exposed to X-ray films.

Transfection of small-interfering RNA (siRNA). Individual siRNA against CHOP and non-targeting control were obtained from Dharmacon RNA Technologies (Lafayette, CO, USA). The best conditions of siRNA application were established beforehand by western blotting and enzyme immunoassays. Cells were plated in 6-well, 12-well, or 24-well plates and grown to 50-70% confluence. After 24 h, the cells were transfected with siRNA (100-200 nmol/l) using lipofectamine-2000 reagent (Invitrogen, Carlsbad, CA, USA) for 48-72 h, according to the manufacturer's instructions.

Promoter activity assay. In 6-well dishes, 10^5 SNU-1041cells were seeded per well and grown to approximately 70% confluence in complete growth media containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco). Plasmid DNA in the amount of 0.5 μg with a *CHOP* promoter construct (generously provided by Dr. Shigeru Takahashi of Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo) and 0.5 μg pSV-beta- galactosidase was co-transfected into the cells in each well under serum-free conditions with Lipofectamin reagent (Invitrogen) according to the manufacturer's instructions. After 6 hours, RPMI-1640 medium was added with 10% FBS, and the cells were incubated for 18 hours. The cells were then incubated under serum starvation condition for an additional 24 hours. After cell lysis with 5× reporter lysis buffer, 30 μl cell extract was mixed with 100 μl luciferase assay reagent (Promega). The activity of luciferase was measured in cellular extracts using a TR717 Microplater Luminometer (Tropix Inc., Bedford, MA, USA) with the Bioluminescent Reporter Gene Assay system (Triopix) according to the manufacturer's instructions. To adjust for differences in transfection efficiencies, the luciferase values were normalized using beta-galactosidase.

Results

The growth-inhibitory effect of celecoxib on the SNU-1041 cell line. To determine the growth-inhibitory effect of celecoxib, SNU-1041 cells were treated with celecoxib and their growth was quantified using the MTT assay. The growth of SNU-1041 cells was inhibited by celecoxib (Figure 1A). With the result of this experiment, the optimal concentration of celecoxib to effectively inhibit the cells was determined as at least 20 μM for further experiments of RT-PCR and western blotting.

The effect of celecoxib on the ER stress pathway at the transcription level in SNU-1041 cell line. To determine whether celecoxib induces the expression of the target genes *CHOP*, *GRP78* and *XBP1* in the ER stress pathway in this HNSCC cell line, RT-PCR was performed after treatment of celecoxib. Celecoxib strongly induced expression of *CHOP*, *GRP78* and *XBP1* at the RNA level at 4 h (Figure 1B). Splice *XBP1* mRNA is an active form in the ER stress pathway. When *XBP1* mRNA is activated and spliced by IRE1 (Inositol-requiring protein 1) kinase, double bands of XBP1 can be manifested in RT-PCR. Celecoxib induced splice *XBP1* mRNA in this cell line (Figure 1B).

The effect of celecoxib on ER stress-induced CHOP protein. We assumed that celecoxib could inhibit this HNSCC cell line through the ER stress pathway. Since CHOP has been reported as a key determinant of apoptosis during ER stress response (14, 15), CHOP expression in SNU-1041 was evaluated to verify whether celecoxib-induced apoptosis is related to the ER stress pathway. Western blotting experiments after treatment with different concentrations of celecoxib, showed a significant up-regulation of *CHOP* after treatment with high concentrations (*i.e.* 35 μM or higher) of celecoxib (Figure 1C).

The activation of CHOP promoter by celecoxib. To determine the effects of celecoxib on activation of CHOP, *CHOP* promoter activity assay was performed. It showed that celecoxib increased the activity of the *CHOP* promoter in SNU-1041 in a dose-dependent manner (Figure 1D).

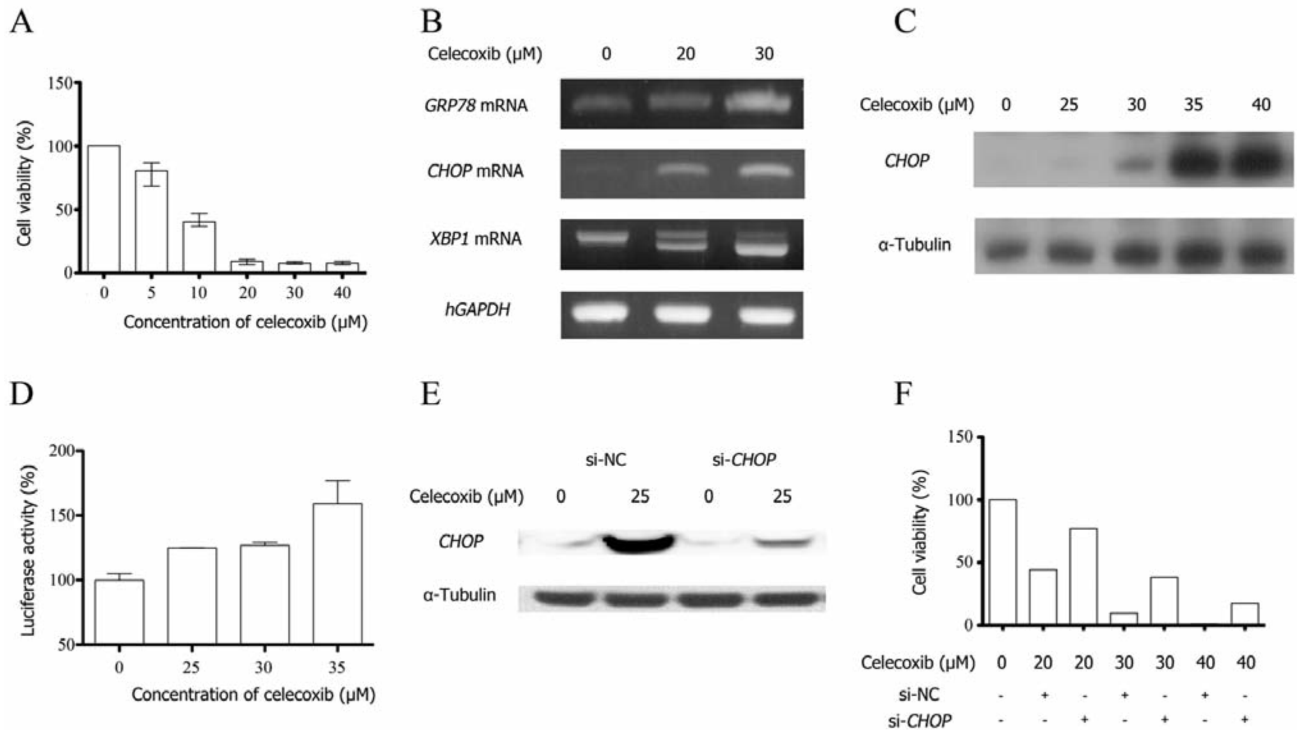

Figure 1. *A: The growth-inhibitory effect of celecoxib. The growth of SNU-1041 cells was inhibited by celecoxib. B: Reverse transcriptase–polymerase chain reaction (RT-PCR). Glucose-regulated protein (GRP78), C/EBP homologous protein (CHOP) and X-box binding protein (XBP1) mRNAs were up-regulated after treatment of celecoxib. When XBP1 mRNA is activated, double bands of XBP1 can be seen in RT-PCR. C: Western blot analysis of CHOP after treatment of celecoxib. The induction of CHOP was apparent after 4 h treatment with high concentrations (35 μM or higher) of celecoxib. D: Promoter activity assay of CHOP. Celecoxib increased the activity of the CHOP promoter in SNU-1041 cells. E: Western blot analysis of CHOP after RNA interference. CHOP siRNA (si-CHOP) and non-target control (si-NC) were transfected into SNU-1041 cells. After 48 h, cells were treated with celecoxib for 18 h. The expression of CHOP by celecoxib was effectively inhibited in the cells transfected with si-CHOP. F: The growth-inhibitory effect of celecoxib in SNU-1041 cells transfected with CHOP siRNA and non-target control siRNA. Inhibition of cell proliferation by celecoxib was effectively hindered with si-CHOP.*

Change in growth-inhibitory effect of celecoxib after inhibition of CHOP. To determine whether *CHOP* siRNA inhibits CHOP production effectively at the transcription level in SNU-1041 cells treated with celecoxib, RT-PCR was performed after transfection of the cells with *CHOP* siRNA (200 nmol/l) and negative control siRNA (si-NC) (200 nmol/l). RT-PCR revealed that celecoxib strongly induced production of *CHOP* mRNA in the control cells transfected with si-NC. However, the expression of *CHOP* mRNA was effectively inhibited by celecoxib in the cells transfected with *CHOP* siRNA (Figure 1E).

To determine whether the inhibition of celecoxib-induced *CHOP* mRNA expression by *CHOP* siRNA affects growth inhibition by celecoxib, the cell proliferation assay was performed after treatment of cells with different concentrations of celecoxib and transfection of si-NC and *CHOP* siRNA. Inhibition of cell proliferation by celecoxib was effectively hindered by *CHOP* siRNA (Figure 1F).

Discussion

Celecoxib triggered the ER stress response in this HNSCC cell line, whose growth was inhibited by celecoxib. This growth inhibition by celecoxib was effectively hindered with the use of *CHOP* siRNA, which blocks the ER stress response. Therefore the ER stress pathway could be a COX-independent anticancer mechanism of celecoxib.

Earlier studies showed that COX2 inhibitors have COX2-independent anticancer effects, although the exact mechanisms have not been clearly identified. Particularly intriguing are several reports describing potent anti-proliferative and proapoptotic effects of celecoxib in the absence of any apparent involvement of COX2 (7, 16-21). Suggested mechanisms include the inhibition of beta-catenin translocation to the nucleus, the activation and inhibition of mitogen-activated protein kinases (MAPKs), blocking Akt activation, induction of phosphodiesterases, activation of

peroxisome proliferation activated receptor, regulation of nuclear factor-κB activity, and induction of NSAID-associated genes. Celecoxib can inhibit cell proliferation through activation of ERK and p38 MAPK in HNSCC (13).

NSAIDs have been reported to increase intracellular calcium levels. The intracellular Ca^{2+} level is involved in NSAID-induced apoptosis, and the ER, which contains high concentrations of Ca^{2+}, plays an important role in intracellular Ca^{2+} homeostasis. Although CHOP, GRP78 and XBP1 are not unique, these are known indicators of the initiation of the ER stress pathway (8-12). As a first step of this study, we confirmed that celecoxib was involved in the activation of the ER stress pathway by identifying effects on these genes at the transcription level.

CHOP is considered as a main determinant of cellular fate and in the acceleration of ER stress-induced apoptosis (14). CHOP is a short-lived protein, so under mild or transient ER stress, CHOP expression is not prolonged and the cell can adapt and recover. However, if ER stress is intense or prolonged, CHOP expression is persistent and cell death ensues (15). In the present study, CHOP was significantly up-regulated after treatment with celecoxib in HNSCC cells. To verify whether CHOP is a factor of apoptosis in response to celecoxib treatment, the RNA interference method was used. *CHOP* siRNA inhibited *CHOP* expression efficiently at the transcription level in SNU-1041 cells treated with celecoxib and effectively blocked the antitumor action of celecoxib.

Although this study was a limited study using a single cell line, we believe the ER stress response is involved in the inhibition of tumor growth by celecoxib. Further investigation into this COX2-independent mechanism of celecoxib is warranted to facilitate clinical use of celecoxib as an anticancer agent.

Conclusion

The ER stress pathway could be a COX-independent anticancer mechanism of celecoxib.

Acknowledgements

This work was supported by Research Grant (No. 0420090730) of Seoul National University Hospital.

References

1 Ulrich CM, Bigler J and Potter JD: Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer *6*: 130-140, 2006.

2 Diperna CA, Bart RD, Sievers EM, Ma Y, Starnes VA and Bremner RM: Cyclooxygenase-2 inhibition decreases primary and metastatic tumor burden in a murine model of orthotopic lung adenocarcinoma. J Thorac Cardiovasc Surg *126*: 1129-1133, 2003.

3 Gupta S, Adhami VM, Subbarayan M, MacLennan GT, Lewin JS, Hafeli UO, Fu P and Mukhtar H: Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. Cancer Res *64*: 3334-3343, 2004.

4 Roh JL, Sung MW, Park SW, Heo DS, Lee DW and Kim KH: Celecoxib can prevent tumor growth and distant metastasis in postoperative setting. Cancer Res *64*: 3230-3235, 2004.

5 Williams CS, Watson AJ, Sheng H, Helou R, Shao J and DuBois RN: Celecoxib prevents tumor growth *in vivo* without toxicity to normal gut: lack of correlation between *in vitro* and *in vivo* models. Cancer Res *60*: 6045-6051, 2000.

6 Patel MI, Subbaramaiah K, Du B, Chang M, Yang P, Newman RA, Cordon-Cardo C, Thaler HT and Dannenberg AJ: Celecoxib inhibits prostate cancer growth: evidence of a cyclooxygenase-2-independent mechanism. Clin Cancer Res *11*: 1999-2007, 2005.

7 Maier TJ, Janssen A, Schmidt R, Geisslinger G and Grosch S: Targeting the beta-catenin/APC pathway: a novel mechanism to explain the cyclooxygenase-2-independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. FASEB *19*: 1353-1355, 2005.

8 Johnson AJ, Hsu AL, Lin HP, Song X and Chen CS: The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca^{2+}-ATPases: A plausible link with its antitumour effect and cardiovascular risks. Biochem J *366*: 831-837, 2002.

9 Kim SH, Hwang CI, Park WY, Lee JH and Song YS: GADD153 mediates celecoxib-induced apoptosis in cervical cancer cells. Carcinogenesis *27*: 1961-1969, 2006.

10 Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M, Katsu T, Suzuki K, Tsutsumi S and Mizushima T: Involvement of intracellular Ca^{2+} levels in nonsteroidal anti-inflammatory drug-induced apoptosis. J Biol Chem *280*: 31059-31067, 2005.

11 Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M and Mizushima T: Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ *11*: 1009-1016, 2004.

12 Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S and Samali A: Targeting the endoplasmic reticulum-stress response as an anticancer strategy. Eur J Pharmacol *625*: 234-246, 2009.

13 Park SW, Kim HS, Hah JW, Jeong WJ, Kim KH and Sung MW: Celecoxib inhibits cell proliferation through the activation of ERK and p38 MAPK in head and neck squamous cell carcinoma cell lines. Anticancer Drugs *21*: 823-830, 2010.

14 Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP and Ron D: CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev *18*: 3066-3077, 2004.

15 Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D and Kaufman RJ: Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol *4*: e374, 2006.

16 Elder DJ, Halton DE, Playle LC and Paraskeva C: The MEK/ERK pathway mediates COX2-selective NSAID-induced apoptosis and induced COX2 protein expression in colorectal carcinoma cells. Int J Cancer *99*: 323-327, 2002.

17 Baek SJ, Wilson LC, Lee CH and Eling TE: Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): Inhibition of cyclooxygenase and induction of NSAID-activated gene. J Pharmacol Exp Ther *301*: 1126-1131, 2002.

18 Kardosh A, Blumenthal M, Wang WJ, Chen TC and Schonthal AH: Differential effects of selective COX2 inhibitors on cell cycle regulation and proliferation of glioblastoma cell lines. Cancer Biol Ther *3*: 55-62, 2004.

19 Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH, Fowble JW, Ward PJ and Chen CS: 3-phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. Cancer Res *64*: 1444-1451, 2004.

20 Tegeder I, Pfeilschifter J and Geisslinger G: Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J *15*: 2057-2072, 2001.

21 Zhang X, Morham SG, Langenbach R and Young DA: Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. J Exp Med *190*: 451-459, 1999.

Received January 2, 2014
Revised February 6, 2014
Accepted February 7, 2014