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

WONJAE CHA1,3, SEOK-WOO PARK2, TACK-KYUN KWON1, J. HUN HAH1,2 and MYUNG-WHUN SUNG1,2

1Department of Otorhinolaryngology–Head and Neck Surgery, Seoul National University Hospital, Seoul, Republic of Korea;
2Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea;
3Department 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.

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/l diethyl dithiocarbamate, 1 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride) and, 24 h later, were treated with CD. After dithiocarbamate, 1 mmol/l EDTA, and 1 mmol/l phenylmethyl-

- **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).
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 antiproliferative 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 Ca2+ level is involved in 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


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