Phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) is a critical convergence point of the integrated stress response (ISR), which supports eukaryotic cellular adaptation to diverse stressful conditions, including the endoplasmic reticulum (ER) stress by global protein translational arrest and induction of numerous stress-triggered cytoprotective genes. Challenge with non-steroidal anti-inflammatory drug (NSAID) leads to ER perturbation that may sensitize cancer cells to drug-induced apoptosis. Here, we examined the ER stress signals in the context of NSAID exposure and the induction of the critical tumor suppressor, NSAID-activated gene 1 (NAG-1), in the epithelial cancer cells. Sulindac sulfide, the active sulindac metabolite, was shown to trigger the ISRs via eIF2α kinase such as RNA-dependent protein kinase-related endoplasmic reticulum kinase (PERK) and RNA-dependent protein kinase (PKR). ER stress markers such as glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP) and activating transcription factor (ATF)-3 were enhanced by sulindac sulfide in colon cancer cells. In these cells, the PERK-activated ATF3–CHOP signaling pathway mediated the gene expression of pro-apoptotic NAG-1 and NSAID-induced apoptosis. In contrast, PKR protein was not involved in the signaling cascade for the gene expression of CHOP-linked NAG-1. Instead, PKR mediated activation of pro-survival extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, which was enhanced by NAG-1 suppression in response to cytotoxic sulindac sulfide exposure. PKR–ERK1/2 activation may thus contribute to the defensive cellular response to cytotoxic NSAIDs while drug-mediated ER stress triggers the pro-apoptotic NAG-1 production in human colon cancer cells.

Abbreviations: ATF, activating transcription factor; CHOP, C/EBP homologous protein; COX, cyclooxygenase; eIF2α, eukaryotic translation initiation factor 2 alpha; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; GI, gastrointestinal; GRP78, glucose-regulated protein 78; ISR, integrated stress response; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; NAG-1, NSAID-activated gene 1; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; PERK, RNA-dependent protein kinase-related endoplasmic reticulum kinase; PKR, RNA-dependent protein kinase.

The integrated stress response-associated signals modulates intestinal tumor cell growth by NSAID-activated gene 1 (NAG-1/MIC-1/PTGF-β)

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to relieve pain and inflammation and have also received considerable attention because of their protective effects against human cancer (1,2). NSAIDs inhibit the enzymatic activity of cyclooxygenase (COX) involved in production of prostaglandins that inhibit apoptosis, stimulate tumor growth and enhance angiogenesis, tumor cell invasion and metastasis in cancer models (3,4). Clinically, the NSAID sulindac represses familial adenomatous polyposis (5). However, resistance to sulindac is observed in familial adenomatous polyposis patients as shown by progression toward the rectal malignancy after prolonged sulindac chemoprevention (6–9). Alternative approaches to overcome sulindac resistance have involved the drug metabolism. Sulindac is metabolized in vivo to its sulfide and sulfone derivatives, of which only sulindac sulfide inhibits COX enzymes (10). NSAIDs can prevent cancer via both COX-dependent and COX-independent mechanisms by triggering antitumor actions such as tumor cell death and suppression of metastasis. Sulindac sulfide induces apoptosis and suppresses tumor cell invasion by diverse COX-independent mechanism (11–14). Some lines of evidence suggested that NSAIDs modulate normal epithelial and tumor cell growth by COX-independent signaling pathways (15–17). Among the COX-independent regulation, NSAIDs induce apoptosis-inducing or growth-arresting proteins such as NSAID-activated gene 1 (NAG-1; also known as MIC-1, PTGF-β, PLAB, GDF15, PDF and PL74) (18,19). NAG-1 is a transforming growth factor-β superfamily cytokine that is involved in epithelial tumor pathogenesis (5,15,20). Under normal resting conditions in epithelial cells, there is little or no detectable expression of NAG-1. Generally, normal resting epithelial cells in various organs such as the gastrointestinal (GI) tract express lower levels of NAG-1 expression. NAG-1 expression is, however, dramatically increased in inflammation, injury and malignancy (21–25). Increased NAG-1 expression is a feature of many cancers, including breast, colon and prostate. Many epithelial tumor cells secrete high amounts of NAG-1, which is also supported by studies for demonstrating major increase of NAG-1 in cancer biopsies (21,24,25). Moreover, serum MIC-1 levels are markedly elevated in the metastatic cancer and particularly in colorectal cancer (26). With epithelial neoplastic transformation, NAG-1 expression rises dramatically and is further increased in response to a variety of antitumorigenic stimuli such as gamma irradiation, anti-inflammatory phytochemicals and NSAIDs. During the early stages of tumorigenesis, anticancer agent-enhanced NAG-1 can lead to tumor cell apoptosis, inhibition of blood vessel formation and tumor cell cycle arrest (11,27–29). These are collectively referred to as chemorepressive activity. NAG-1 can be induced either in a p53-dependent or in a p53-independent way, and its inducible cellular signals also include other diverse growth-regulatory triggers (19,27,30).

Phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) is a highly conserved point of convergence for the distinct signaling pathways that adapt eukaryotic cells to diverse stressful conditions (31,32). It provides stress resistance by global protein translation arrest and induction of numerous stress-triggered cytoprotective genes. eIF2α phosphorylation-dependent, stress-inducible cellular responses are collectively known as the integrated stress response (ISR) and four different kinases are known to phosphorylate eIF2α. Endoplasmic reticulum (ER) proximal sensor such as RNA-dependent protein kinase-related endoplasmic reticulum kinase (PERK) represses the global translation by phosphorylation of eIF2α, which limits the cellular supply for protein translation and provides cells with sufficient time to fix misfolded proteins from the ER stress. Along with PERK, cells also have the additional mammalian eIF2α kinases, including RNA-dependent protein kinase (PKR), general control non-derepressible-2 (GCN2) and heme-regulated inhibitor (HRI), depending on the types of cellular stress (32). Intense and persistent translational arrest via phosphorylation of eIF2α during ISR will trigger pro-survival or pro-apoptotic signals, depending upon the pathological environment and intracellular tolerance to the limited productivity of the protein factories. One of the critical pro-survival proteins is glucose-regulated protein 78 (GRP78/Bip), which facilitates protein folding, assembly and ER calcium ion (Ca2+) binding as a central ER-resident sensor (33). However, excessive and prolonged deleterious stress during ISR leads to the apoptotic cell death that is associated with a range of inflammatory, carcinogenesis and metabolic diseases. Although the precise molecular mechanisms of ISR-associated apoptosis are unknown, one of the representative factors involved in ISR-linked apoptosis is C/EBP homologous protein (CHOP), whose deficiency in vivo causes the resistance to ER.
stress-induced cell death (34). As a possible target for cellular responses to the NSAID, ER stress-associated apoptotic responses have been implicated in the recent reports (35,36).

In the present study, we evaluated the potential regulation of NAG-1 by NSAID-induced ISR including ER stress response in colorectal epithelial cancer cells. We investigated the effects of tumor-associated NAG-1 expression and its regulatory signaling pathways induced by sulindac sulfide exposure. As well, potential roles of NAG-1 induction in association with ER stress were addressed in terms of the cellular stress pathways leading to cancer cell death or survival. The goal of this study was to provide mechanistic insight into tumor cell responses to NSAID-induced ER stress.

Materials and methods

Cell culture conditions and reagents

Intestinal cancer cell lines, HCT-116, HT-29, intestine-407 and HCT-8, were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich, St Louis, MO), 50 U/ml penicillin (Sigma–Aldrich) and 50 µg/ml streptomycin (Sigma–Aldrich) in a 5% CO2 humidified incubator at 37°C. HCT-116 cell line has been extensively investigated as a representative model of human colon cancer in culture (37,38). Non-transformed intestinal epithelial cell line IEC-18 was purchased from Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS (Sigma–Aldrich), 50 U/ml penicillin (Sigma–Aldrich), 50 µg/ml streptomycin (Sigma–Aldrich) and 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid. Cell number and viability were assessed by exclusion of trypan blue dye (Sigma–Aldrich) using a hemocytometer. Antibodies were purchased from the indicated companies as rabbit polyclonal anti-activating transcription factor (ATF)-3, mouse monoclonal anti-p-ERK1/2, mouse monoclonal anti-poly-ADP ribose polymerase (PARP)-1, mouse monoclonal anti-CHOP (GPCD153), goat polyclonal anti-NAG-1 (PTGF-R), rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-p-eIF2α (Assay Designs, Ann Arbor, MI), polyclonal anti-extracellular signal-regulated kinase (ERK1/2) antibody and rabbit polyclonal anti-p-eIF2α (Cell Signaling Technology, Beverly, MA). Expression plasmid for short hairpin RNA of NAG-1 (pShNAG-1), dominant-negative CHOP (dnCHOP), dominant-negative PERK (dnPERK) and dominant-negative PKR (dnPKR) were kindly provided by Dr Thomas Eling (National Institute of Environmental Health Sciences, Research Triangle Park, NC), Dr Tomomi Gotoh (Kumamoto university, Japan) (39), Dr J Alan Diehl (Abramson Family Cancer Research Institute) or Pei-Jer Chen (National Taiwan university, Taiwan), respectively. Expression vector for dominant-negative mitogen-activated protein kinase/extraacellular signal-regulated kinase kinase 1 (MEK1) (dnMEK1) was purchased from Bionym Technology (San Diego, CA). Luciferase reporter containing NAG-1 promoter (~1739/+70) was kindly provided from Dr Eling.

Assessment of apoptosis by fluorescence-activated cell sorting

The DNA content of cells was determined by fluorescence-activated cell sorting analysis. Cells were plated in triplicate two or more times at 7.5 × 10^4 cells per well in 60 mm diameter plates, incubated overnight and then treated with serum-free media for 24 h. After treatment, the cells were rinsed with phosphate-buffered saline (PBS), harvested, mixed with annexin V–fluorescein isothiocyanate in the dark for 15 min, centrifuged and incubated with propidium iodide (Sigma–Aldrich) according to the instructions of the manufacturer. Fluorescence-activated cell sorting of 7500 cells utilized FACsaria apparatus (Becton Dickinson, Franklin Lakes, NJ) equipped with CellQuest software by gating on an area versus width dot plot to exclude cell debris and cell aggregates. Apoptosis was measured by the level of subdiploid DNA content in cells using CellQuest software from the total gated cells.

DNA fragmentation analysis

DNA was extracted from a colon epithelial cell line. In brief, 2 × 10^6 cells suspended in PBS were centrifuged for 5 min, 2000 r.p.m. for 4°C, and the pellet was suspended in 0.1 ml hypotonic lysis buffer (10 mM Tris, pH 7.4, 10 mM ethylenediaminetetraacetic acid, pH 8.0, and 0.5% vol/vol Triton X-100). Cells were incubated for 15 min at 4°C. The resultant lysate was centrifuged for 30 min, 13 000 r.p.m., 4°C. The supernatant containing fragmented DNA was digested for 1 h at 37°C with 0.04 mg/ml of RNase A (Bio Basic, Markham, Ontario, Canada) and then incubated for an additional 1 h at the same temperature with 0.04 mg/ml of protease K (Sigma–Aldrich). DNA was precipitated in 50% (vol/vol) isopropanol in 0.4 M NaCl at –20°C overnight. The precipitate was centrifuged at 13 000 r.p.m. for 30 min at 4°C. The resulting pellet was air-dried and resuspended in Tris–EDTA buffer. An aliquot equivalent to 2 × 10^5 cells was electrophoresed at 40 V for 3 h in a 2% (wt/vol) agarose gel in 90 mM Tris–glacial acetic acid buffer containing 2 mM ethylenediaminetetraacetic acid (pH 8.0). After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) and the nuclei bands were visualized with an ultraviolet transilluminator. A 1 kb DNA ladder (SolGent, Daejeon, Korea) was used for molecular sizing.

Western immunoblot analysis

Levels of protein expression were compared using western immunoblot analysis. Cells were washed with ice-cold PBS, lysed in boiling lysis buffer [1% (wt/vol) sodium dodecyl sulfate, 1.0 mM sodium ortho-vanadate and 10 mM Tris, pH 7.4] and sonicated for 5 s. Protein in the lysate was quantified using a BCA protein assay kit (Pierce, Rockford, IL). Fifty micrograms of protein were separated by mini gel electrophoresis (Bio-Rad, Hercules, CA). The proteins were transferred onto a polyvinylidene difluoride membrane (Amerham Pharmacia Biotech, Piscataway, NJ, USA). The blot was blocked for 1 h with 5% skim milk in Tris-buffered saline plus Tween 0.05% and probed with the primary antibody for 2 h at room temperature or overnight at 4°C. After washing three times with Tris-buffered saline plus Tween 0.05%, the blot was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and then washed three times with Tris-buffered saline plus Tween 0.05%. Protein was detected using enhanced chemoiluminescence substrate (Amerham Pharmacia Biotech).

Traditional reverse transcription–polymerase chain reaction

RNA was extracted with RNeasy kit (Qagen, Valencia, CA) according to the manufacturer’s instructions. RNA (100 ng) from each sample was transcribed to complementary DNA by BD Sprint PowerScript (Clontech, Mountain View, CA). The amplification was performed with Hot Start ExTaQ DNA polymerase (Takara Bio, Shiga, Japan) in a Mycycler thermal cycler (Bio-Rad) using the following parameters: denaturation at 94°C for 2 min and 25 cycles of denaturation at 98°C for 10 s, annealing at 59°C for 30 s and elongation at 72°C for 45 s. An aliquot of each polymerase chain reaction product was subjected to 1.2% (wt/vol) agarose gel electrophoresis and visualized by staining with ethidium bromide. The forward and 3′ reverse-complement polymerase chain reaction primers for amplification of each gene were as follows: human GRP78 (5′-TGACGAGGACATCAAGTTGC-3′ and 5′-CGCTGGTCAAGGCTTCC-3′), human CHOP (5′-CCGGGCTGCTGAGGGAGG-3′ and 5′-TCACCACTCGGTGTTACAGA-3′), human GADD34 (5′-GAATCCAGCGCAAGGAAATG-3′ and 5′-AGGGAGACAC-TGCAGTCTC-3′), human MIC-1 (5′-AGCTCGAGGAGACACG-3′ and 5′-GAGATCCGCGAGATGTTG-3′), human ATF4 (5′-CCGACCGTTGAGATCAG-3′ and 5′-GACCAGCATCACAGATTGTAC-3′), human ATF3 (5′-CCCTTGTGCTACCTGGTTT-3′ and 5′-AGGACCTCCGCTTCTGTT-3′), human ATF5 (5′-GATCTCGAGGAGACACG-3′ and 5′-AGGACGTCCGCTTCTGTT-3′) and human glyceraldehyde 3-phosphate dehydrogenase (5′-TCAACGGGCCGTTGTAAT-3′ and 5′-CTGGTGCTAGACCCAATCTC-3′). Relative amount of each messenger RNA was quantified by dividing by density of house keeping gene.

Transient and stable transfection

Cells were transfected with a mixture of plasmids using Trans-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer’s protocol. For transfection of the luciferase reporter gene, a mixture of 1.5 µg firefly luciferase reporter and 0.15 µg Renilla luciferase, pRL-null vector (Promega, Madison, WI) per 4.5 µl of Trans-LT1 reagent was applied to wells of a six-well culture plate. For the luciferase assay, at 18 h after transfection, cells were exposed to chemicals for the next 24 h and lysed for dual-luciferase reporter assay system (Promega). Transfection efficiency was maintained at ~50 to 60%, which was confirmed with pMX-enhanced green fluorescent protein vector. To create pSilence- and pSilEGR1-expressing stable cell lines, cells were transfected using Trans-LT1 reagent. After 48 h, the cells were subjected to selection for stable integrants by exposure to 500 µg/ml G418 (Invitrogen) in complete medium containing 10% FBS. Selection was continued until monolayer colonies formed. The transfectants were then maintained in medium supplemented with 10% FBS and 250 µg/ml G418.

Luciferase assay

Cells were washed with cold PBS, lysed with passive lysis buffer (Promega) and then centrifuged at 12 000g for 4 min. The supernatant was collected, isolated, and stored at –80°C until assessment of luciferase activity. Luciferase activity was measured with a dual-mode luminometer (Model TD-20/20; Turner Designs, Sunnyvale, CA) after briefly mixing the supernatant (10 µl) with 50 µl firefly luciferase assay substrate solution, followed with 50 µl stopping Renilla luciferase assay solution (Promega). Luciferase activity was normalized by dividing firefly luciferase activity by Renilla luciferase activity.
Fig. 1. Effects of sulindac sulfide on ER markers. (A) Vector-, dominant-negative PKR expression vector (dnPKR)- or dominant-negative PERK expression vector (dnPERK)-transfected HCT-116 cells were treated with 25 μM sulindac sulfide for 10 min and total cell lysates were analyzed by western blot. (B and C) HCT-116 cells were treated with 25 μM sulindac sulfide (B) or 100 μM indomethacin (C) for the indicated times. (D and E) HT-29 cells (D) and intestine 407 cells (E) were treated with 25 μM sulindac sulfide for the indicated times. Total RNA was extracted and analyzed using reverse transcription–polymerase chain reaction method. All results are representative of three independent experiments.
Cell survival analysis

One thousand cells were plated on a 60 mm diameter dish for 15 days for focus formation assays. First, cells were exposed to NSAID for 48 h and incubated for 12 days at 37°C in the NSAID-free complete medium in a fully humidified atmosphere of 5% CO₂ in air. Cells were then washed with PBS, fixed with 100% methanol, stained and visualized with Karyomax Giemsa solution (Invitrogen) for 2 min followed by successive washes with PBS at room temperature to visualize colony growth. Cells were counted from 10 randomly selected 2 cm² grids per well.

Statistical analyses

Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student’s t-test was performed. For comparison of multiple groups, data were subjected to analysis of variance and pairwise comparisons made by the Student–Newman–Keuls method. Data not meeting normality assumption were subjected to Kruskal–Wallis analysis of variance on ranks and then pairwise comparisons were made by the Student–Newman–Keuls method.

Results

Sulindac sulfide triggers ER stress signals in HCT-116 colon cancer cells

Global translational inhibition in ISR was observed by measuring eIF2α phosphorylation in the colon cancer cells. PERK represses the translation by phosphorylation of eIF2α, allowing cells to have sufficient times to fix the misfolded proteins from the ER stress. Sulindac sulfide activated the phosphorylation of eIF2α that was suppressed by dominant-negative PERK expression (Figure 1A). PKR is another kind of eIF2α kinase whose repression using dominant-negative form also alleviated the enhanced phosphorylation of eIF2α (Figure 1A). In addition to eIF2α phosphorylation, the ER stress-linked gene expression profile was analyzed in the drug-exposed colon cancer cells. In response to sulindac sulfide, pro-survival factor GRP78 and pro-apoptotic CHOP protein were assessed in HCT-116 colon cancer cells. Sulindac sulfide increased gene expression of GRP78 and CHOP (Figure 1B). Moreover, other CHOP-regulating products such as central signaling mediator ATF3 and ATF4 and CHOP-mediated product GADD34 were also elevated by sulindac sulfide treatment. In response to ISR in the mammalian cells, ATF3, a member of the ATF/cyclic adenosine 3’5’-monophosphate response element binding subfamily of the basic-region leucine zipper (bZIP) family, plays an integral role in the coordination of gene expressions (40). Another NSAID indomethacin was also shown to trigger gene expression of CHOP and CHOP-mediating products (Figure 1C). Moreover, similar expression pattern of ISR-related signaling molecules (CHOP, GRP78 and ATF3) were observed in other intestinal tumor cell lines, including HT-29 and intestine 407 cells (Figure 1D and E). In summary, indicators of the global translational arrest such as eIF2α phosphorylation and ER stress indicators such as pro-apoptotic CHOP-linked signaling molecules were elevated in NSAID sulindac sulfide-exposed human intestinal tumor cells.

Pro-apoptotic NAG-1 is elevated by PERK-linked ER stress signals in HCT-116 colon cancer cells

As a critical tumor-suppressive mediator, NAG-1 has been investigated in diverse tumor models (41–43). In the present study, NAG-1 was assessed for its pro-apoptotic activity in HCT-116 colon cancer cells. When compared with pro-drug sulindac and its sulfone derivative at the same dose regime, only sulindac sulfide increased NAG-1 expression. Moreover, sulindac sulfide was also strong inducer of pro-apoptotic CHOP and pro-survival GRP78 expression in human colon cancer cells (Figure 2A). These patterns are similar with the fact that COX is efficiently inhibited only by sulindac sulfide among sulindac metabolites. Sulindac sulfide-induced NAG-1 expression was also confirmed in the other intestinal cancer cell HCT-8 (Figure 2B). On the assumption that enhanced NAG-1 might mediate drug-induced apoptosis, the ability of sulindac sulfide to induce apoptotic cell death via NAG-1 induction was assessed. Sulindac sulfide increased apoptotic DNA fragmentation in HCT-116 cells, which was attenuated in NAG-1-repressed cells, suggesting positive regulation of apoptosis by NAG-1 (Figure 2C).

Since sulindac sulfide triggered ER stress-related signals, we investigated the involvement of ER stress in NAG-1 production. PERK, a critical eIF2α kinase, activates pro-apoptotic ER stress mediators. When PERK was blocked using its dominant-negative form,
pro-apoptotic CHOP and NAG-1 protein were suppressed in the colon cancer cells (Figure 3A). At the integral point of the eIF2α kinase stress response, ATF3 plays critical roles in mediating CHOP expression. Sulindac sulfide enhanced ATF3 gene expression, which was attenuated by PERK suppression (Figure 3A), and enforced expression of antisense ATF3 reduced the pro-apoptotic gene expression of CHOP and NAG-1 in the colon cancer cells (Figure 3B). To determine if CHOP as a transcription factor can affect gene expression of downstream mediators, dominant-negative CHOP protein was tested for its effects on NAG-1. NAG-1 protein and promoter activity were suppressed by dominant-negative CHOP expression (Figure 3C and D). CHOP was thus a positive regulator of NAG-1 induction by sulindac sulfide. Data were also consistent with direct contribution of NAG-1 and CHOP to sulindac sulfide-mediated apoptotic death in HCT-116 colon cancer cells.

PKR contributes to the activation of ERK1/2-linked survival signals in response to the cytotoxic sulindac sulfide

Other than the PERK-linked pro-apoptotic signals, PKR was also involved in translational arrest by sulindac sulfide (Figure 1A). However, induction of pro-apoptotic CHOP and NAG-1 was not repressed by expression of dominant-negative form of PKR (Figure 5A). Moreover, PKR was not positively associated with sulindac sulfide-mediated apoptotic cell death (Figure 5B). Instead, PKR suppression increased the cancer cell apoptosis, implicating pro-survival effects of PKR in NSAID-induced cell death. PKR suppression also elevated the cleavage of PARP in the presence of sulindac sulfide (Figure 5C). In the survival test, PKR-suppressed cells showed less survival from...
Fig. 4. Effects of NAG-1 on sulindac sulfide-mediated apoptosis. (A) Vector, NAG-1 short hairpin RNA expression plasmid (pShNAG-1) or dominant-negative CHOP expression vector (dnCHOP)-expressing HCT-116 cells were treated with 25 μM sulindac sulfide and apoptosis was quantified using the annexin V/fluorescein isothiocyanate staining method. (B and C) Transfected cells were treated with 25 μM sulindac sulfide and total cell lysates were subjected to western blot analysis. All results are representative of three independent experiments.

Fig. 5. Role of PKR in sulindac sulfide-induced cell death. (A) Vector- or dnPKR-expressing cells were treated with 25 μM sulindac sulfide for 24 h and total cell lysates were subjected to western blot analysis. (B) Vector- or dominant-negative PKR plasmid (dnPKR)-expressing HCT-116 cells were treated with 25 μM sulindac sulfide and apoptosis was quantified using the annexin V/fluorescein isothiocyanate-staining method. (C) Vector- or dnPKR-expressing cells were treated with 25 μM sulindac sulfide and total cell lysates were subjected to western blot analysis. (D) Vector- or dnPKR-expressing HCT-116 cells were treated with 25 μM sulindac sulfide and the survival test was performed in accordance with the methods described. All results are representative of three independent experiments.
the cytotoxic exposure to sulindac sulfide than the empty vector-transfected HCT-116 cells (Figure 5D). As a survival factor in sulindac sulfide-induced apoptosis, ERK1/2 mitogen-activated protein kinase was analyzed in the colon cancer cells. MEK–ERK1/2 pathway was blocked with specific MEK1 inhibitor or dominant-negative MEK1 expression, which increased cellular apoptosis and cleavage of pro-apoptotic PARP protein in the presence of sulindac sulfide (Figure 6A–C). When NAG-1 induction by sulindac sulfide was retarded by NAG-1 short hairpin RNA expression, activation of the survival kinase ERK1/2 was more enhanced, suggesting a negative regulation of ERK1/2 by pro-apoptotic NAG-1 protein (Figure 6D). In contrast, survival factor ERK1/2 was positively linked with PKR signal in response to the cytotoxic sulindac sulfide (Figure 6E). These observations were consistent with the suggestion that pro-survival PKR mediates ERK1/2 activation, which may contribute to the defensive cellular response to the pro-apoptotic NAG-1 production and ER stress responses in drug-exposed human colon cancer cells.

**Discussion**

The present study demonstrates that the NSAID sulindac sulfide triggers ER stress in colon epithelial cancer cells, which mediates the induction of pro-apoptotic NAG-1, whereas cells display survival-related responses such as PKR-associated ERK activation in response to the cytotoxic action of the drug (Figure 7). NAG-1 has been suggested to play a role in the antitumor activity of chemopreventive agents like NSAIDs and anti-inflammatory natural products.

In this study, NAG-1 was identified as a critical factor in sulindac sulfide-induced cell death in the context of the integrated ER stress response and survival. The schematic signaling patterns illustrate that NSAID-induced pro-apoptotic NAG-1 protein is modulated by ER stress signals such as PERK, ATF3, and CHOP protein, which is associated with drug-induced apoptotic cell death. In contrast, the cytotoxic action of NSAID can enhance PKR-regulated ERK survival signals in the colon cancer cells.
response. The ER stress marker CHOP was demonstrated not only to be a critical inducer of NAG-1 expression but also to be a trigger of drug-induced apoptosis by inducing as-yet unknown downstream mediators in the colon cancer cells. Three target genes of CHOP referred to as DOCs (downstream of CHOP) have been identified (44,45). They are carbonyl anhydrase VI, a Temn/Odz homologue and actin-binding protein. However, none is directly involved in death or survival responses. The present study is the first report implicating CHOP-regulated positive death factor NAG-1 in the mediation of ER stress-induced apoptosis in the colon cancer cells. Recent study demonstrates that exogonous expression of NAG-1 protein enhances death receptors such as death receptor4 and DR5 in the gastric cancer cells (46). In turn, the DR pathway activates caspase-3-dependent PARP cleavage and DNA fragmentation. Moreover, ER stress-induced CHOP mediates apoptotic cell death by enhancing DR5 gene expression (47). Since ER stress-induced DR5 expression is p53 independent, ER stress-triggering therapeutics such as NSAIDs could be used in combination with treatment with antitumor tumor necrosis factor—related apoptosis-inducing ligand against human cancers with p53 mutation. ER stress-induced CHOP enhances NAG-1 gene expression as well as DR5. However, we cannot exclude the possibility that pro-apoptotic NAG-1 can be indirectly induced by CHOP protein. Further studies designed to demonstrate the direct involvement of CHOP transcription factor in NSAID-induced NAG-1 transcriptional activation are required. Despite the antitumor activity of NSAIDs, NAG-1 can be associated with the drug-induced cytotoxic effects in the normal GI epithelial tissues since NAG-1 was also induced in the non-transformed intestinal IEC-18 cells (data not shown). NSAIDs have been well recognized to cause severe ulcers in the upper GI tract (48,49). Consequences include increased mucosal permeability, mucosal inflammation, anemia and occult blood loss, malabsorption, protein loss, ileal dysfunction, diarrhea, mucosal ulceration, strictures due to diarrhagm disease, active bleeding and perforation. Since pro-apoptotic CHOP and NAG-1 can play key roles in NSAID-induced epithelial cytotoxicity, the net efficacy of integrated ER stress-modulating NSAIDs in the cancer patients needs to be reassessed. Our treatment concentrations of sulindac sulfide agree with the dose levels reported in the other study, which is measured up to 50 μM in the human plasma depending on the treatment regime (50). Moreover, sulindac sulfide is locally concentrated in the GI epithelium to levels that are at least 20-fold higher than those achieved in serum (51). Therefore, epithelial exposure levels and the consequent cytotoxicity could be higher than expected.

In response to the cytotoxic sulindac sulfide, cells also can produce the survival signals. Tumor cells can be resistant to NSAID-induced apoptosis via production of death factors such as CHOP and NAG-1. The balance between the cytotoxic response and defensive survival will determine the cellular fate in the context of tumor environment upon the NSAID exposure. In this study, PKR-triggered ERK1/2 was the positive growth regulator against the pro-apoptotic signals in the colon cancer cells. A range of cellular stresses including arsenite, ER stressor thapsigargin and hydrogen peroxide can activate PKR as a crucial mediator of the ISR (52). In the present study, PKR was also involved in sulindac sulfide-induced translational arrest but did not contribute to stress-triggered cell death. Instead, PKR was a positive modulator of ERK signals linked with cellular survival response to cytotoxic drugs. PKR is also required for activation of mitogen-activated protein kinases including ERK1/2 in other chemical- or virus-treated mammalian cells, which leads to pro-inflammatory response and growth modulation (53,54). This growth-promoting signaling pathway can enhance the chance of tumor cell survival from the cytotoxic stress of therapeutic NSAIDs. Chemotherapeutic success hinges on the ability to maintain drug-induced tumoricidal activity while suppressing the survival signals in the cancer cells. In contrast, in the normal epithelial cells, the survival signals can contribute to wound healing from NSAID-mediated mucosal injuries in the gut (55–57). Intestinal epithelial injury activates other protective mediators such as ERK1/2 signals. Target genes in ERK1/2-mediated downstream can be involved in the mitogenic and wound healing process (58,59). One of the strong target candidate of ERK1/2 survival signals is early growth response gene 1 (EGR-1). Recent report also suggested protective role of EGR-1 in sulindac sulfide-induced cytotoxicity (60). Sulindac sulfide enhances EGR-1 expression via ERK1/2 signaling pathway, which attenuates NSAID-induced epithelial cytotoxicity. Some other report also suggested involvement of ERK1/2-activated thrombospondin-1 in epithelial survival responses (61). However, recovery of injured tissue via the mitogenic signals is not always beneficial to the body. Chronic accumulation of the mitogenic hits can be a critical cause of the epithelial tumorigenesis (62). Moreover, chronic insult with the mitogenic signals of ERK1/2 pathways has been linked to the oncogenic stimulation and promotion of epithelial tumorigenesis. Considering the mitogenic activity of PKR-linked ERK1/2 signals, much careful examination should be made in terms of the assessment of the cancer-preventive action of NSAID.

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