Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 563 (2007) 49–60
www.elsevier.com/locate/ejphar

Indomethacin induces apoptosis in 786-O renal cell carcinoma cells by activating mitogen-activated protein kinases and AKT

Yen-Chuan Ou [a], Chi-Rei Yang [a], Chen-Li Cheng [a], Shue-Ling Raung [b], Yu-Yeh Hung [a], Chun-Jung Chen [b,c,d,*]

[a] Division of Urology, Taichung Veterans General Hospital, Taichung 407, Taiwan
[b] Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan
[c] Institute of Medical Technology, National Chung-Hsing University, Taichung, 407, Taiwan
[d] Center for General Education, Tunghai University, Taichung 407, Taiwan

Received 17 November 2006; received in revised form 24 January 2007; accepted 24 January 2007
Available online 8 February 2007

Abstract

Studies on chemoprevention of cancer are generating increasing interest. The anti-neoplastic effect of nonsteroidal anti-inflammatory drugs (NSAIDs) involves cyclooxygenase (COX)-dependent and COX-independent mechanisms. Evidence suggests that mitogen-activated protein kinases (MAPKs) may mediate apoptotic signaling induced by anti-neoplastic agents. While many reports have revealed the existence of MAPK activation in apoptosis induced by various stimuli, the signaling transduction pathways used by NSAIDs to trigger apoptosis in human renal cell carcinoma (RCC) remain largely unknown. Treatment of RCC 786-O cells with indomethacin resulted in growth regression and apoptosis. Caspase-dependent apoptosis was evidenced by the detection of enzymatic activities of caspase-3, caspase-6, and caspase-9 and suppression of toxicity using a caspase inhibitor. Indomethacin treatment was associated with increased expression of glucose-regulated protein 78 (GRP78) and C/EBP homologus protein (CHOP) and activation of ATF-6, characteristics of endoplasmic reticulum stress. In addition, the concomitant induction of peroxisome proliferator-activated receptor (PPAR), especially PPAR-β, was apparent in treated cells. Western blotting revealed the activation of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK) with indomethacin treatment. Selective inhibitors of ERK, p38 MAPK, and JNK suppressed the induction of GRP78, CHOP, and PPAR-β, attenuated indomethacin-induced cytotoxicity and reduced increased caspase activity. LY294002, a phosphoinositide-3 kinase (PI3K)/AKT inhibitor, and Trolox, an antioxidant, suppressed indomethacin-induced cytotoxicity and caspase activation. Furthermore, Trolox attenuated indomethacin-induced increased phosphorylation in ERK, p38 MAPK, JNK, and AKT. In conclusion, our findings establish a mechanistic link between the oxidative stress, PI3K/AKT pathway, MAPK pathway and indomethacin-induced cellular alterations and apoptosis in 786-O cells.

Keywords: Apoptosis; Endoplasmic reticulum stress; Indomethacin; MAPK; Oxidative stress; PPAR; Renal cell carcinoma

1. Introduction

Cyclooxygenase (COX), including COX-1 and COX-2, is the rate-limiting enzyme for the production of eicosanoids. In contrast to the constitutively low level expression of COX-1 in most tissues, COX-2 is absent from most normal tissues but is expressed in response to a diverse spectrum of stimuli, including tumor promoters. In particular, the association between COX-2 overexpression and clinico-pathological parameters of aggressiveness and poor outcome has been documented in the vast majority of human cancers (Eberhart et al., 1994; Dubois et al., 1998; Fosslien, 2000). Specifically, overexpression of COX-2 is able to cause cell transformation and induce tumorigenesis in transgenic animals (Liu et al., 2001, 2005). These biological functions of COX-2 imply that it is worth investigating as a potential target for prevention and treatment of human cancers.

* Corresponding author. Department of Education and Research, Taichung Veterans General Hospital, No. 160, Section 3, Taichung-Gang Road, Taichung 407, Taiwan. Tel.: + 886 4 23592525x4022; fax: +886 4 23592705.
E-mail address: cjchen@vghtc.gov.tw (C.-J. Chen).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.01.071

Nonsteroidal anti-inflammatory drugs (NSAIDs), a class of compounds that block eicosanoid production through the inhibition of COX activity, are frequently used in the treatment of diseases because of their analgesic and anti-inflammatory activities (Dubois et al., 1998). Currently, NSAIDs have an emerging utility as chemotherapeutics for the prevention and treatment of cancers. Epidemiological observations suggest that long-term NSAID usage is associated with a reduced incidence of certain cancers (Marnett, 1992; Duperron and Castonguay, 1997; Bakhle, 2001). Experimental evidence has indicated the chemopreventive and therapeutic effects of COX-2 inhibition in COX-2 overexpressed cells such as hepatocellular carcinoma and colon carcinoma (Martin et al., 2005; Kern et al., 2006). However, the chemopreventive and anti-tumorigenic activities of NSAIDs have also been observed in COX-2-deficient cells (Hanif et al., 1996). It remains unclear how NSAIDs exert their anti-neoplastic effects, but apoptotic induction is one of the possible mechanisms.

Selective (inhibition of COX-2) and non-selective (inhibition of COX-1 and COX-2) NSAIDs, including indomethacin, have been shown to exert apoptotic effects in a variety of cells. Apoptotic induction by NSAIDs is demonstrated in esophageal cancer cells, liver cancer cells, colon cancer cells, lung cancer cells, oral cancer cells, and bladder cancer cells dependently of COX-2 or independently of COX-2 through induction of phosphatase and tensin homologue deleted from chromosome ten (PTEN), p53, C/EBP homologus protein (CHOP), peroxisome proliferator-activated receptor gamma (PPAR-γ), or $p75^{NTR}$ tumor suppressor protein (Souza et al., 2000; Wick et al., 2002; Ho et al., 2003; Saito et al., 2003; Chu et al., 2004; Khwaja et al., 2004; Tsutsumi et al., 2004; Martin et al., 2005; Kern et al., 2006). Investigations of the signal transduction pathways responsible for such apoptotic mediator induction leading to cell survival or apoptosis have focused on the mitogen-activated protein kinase (MAPK) and AKT/protein kinase B pathway. Intracellular, MAPKs and AKT are the major oxidative stress-sensitive signal transduction pathways (Shi et al., 2003; Carvalho et al., 2004). It has been recognized that the activation of the p38 MAPK or c-Jun N-terminal kinase (JNK) pathway is involved in stress stimuli-induced apoptosis, whereas extracellular signal-regulated kinase (ERK) and AKT have been shown to protect cells from apoptosis (Xia et al., 1995; Datta et al., 1997; Garrington and Johnson, 1999; Panka et al., 2001). Evidence suggests that the activation of JNK may mediate apoptotic signaling induced by indomethacin in leukemic cells (Zhang et al., 2003).

The actions of NSAIDs against cancers are largely achieved by the induction of apoptosis. However, the action mechanisms of NSAIDs vary and depend on cell types and environmental situations. In human renal cell carcinoma (RCC), an elevated expression of COX-2 is detected in several RCC cell lines (Chen et al., 2004). The expression of COX-2 in RCC is found to correlate with tumor proliferation and angiogenesis (Miyata et al., 2003). While many reports have revealed the existence of MAPK and AKT signaling molecules in apoptosis induced by various stimuli, the signaling transduction pathways used by NSAIDs to trigger apoptosis in human RCC, including the potential role of MAPKs and AKT and their mutual interaction, remain largely unknown. In this study, we tried to determine whether MAPKs and AKT are activated during indomethacin-induced RCC apoptosis and, if this proved to be the case, to identify the role of such activation and the potential downstream effectors.

2. Materials and methods

2.1. Cell culture

Human RCC cell line, 786-O (ATCC CRL1932), were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 100 U/ml penicillin (Gibco Life Technologies) and 100 μg/ml streptomycin (Gibco Life Technologies), and were maintained in a humidified incubator with 5% CO_2.

2.2. Cell viability

Cell viability was assessed by the measurement of formazan production after the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS, Promega, Madison, WI, USA). The number of surviving cells after treatment was determined by measurement of the A_{490} nm of the dissolved formazan product after the addition of MTS for 1 h according to the manufacturer's instructions.

2.3. Flow cytometry assay

The cell cycle distribution was analyzed by flow cytometry (Chen and Liao, 2002). Briefly, cells were trypsinized, washed with PBS, and fixed in 80% ethanol. They were then washed with PBS, incubated with 100 μg/ml RNase at 37 °C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer. The percentage of cells in different phases of the cell cycle was analyzed using Cell-FIT software.

2.4. DNA agarose gel electrophoresis

DNA gel electrophoresis was performed as previously reported (Chang et al., 2005). Briefly, cells were lysed in 0.5% Triton X-100, 5 mM Tris–HCl (pH 7.4), and 20 mM EDTA at 4 °C for 30 min. After centrifugation, the supernatants were extracted with phenol/chloroform and precipitated in ethanol. The resultant DNA (10 μg) was separated on a 1.5% agarose gel and stained with ethidium bromide.

2.5. Western blot

Cells were washed twice with PBS and harvested in Laemmli SDS sample buffer. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad, Richmond, CA). Protein extracts were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes

(Amersham Pharmacia Biotech). Membranes were first incubated with 5% nonfat milk in PBS for 1 h at room temperature to reduce nonspecific binding. Membranes were washed with PBS containing 0.1% Tween-20 (PBST), and then incubated for 1 h at room temperature with the indicated antibodies including caspase-3 (1:1000, Santa Cruz Biotechnology), calnexin (1:3000, Santa Cruz Biotechnology), heat shock protein 70 (HSP70) (1:2000, Zymed), protein disulfide isomerase (PDI) (1:1000, Santa Cruz Biotechnology), glucose-regulated protein 94 (GRP94) (1:1000, Santa Cruz Biotechnology), GRP78 (1:1000, Santa Cruz Biotechnology), CHOP (1:1000, Sigma), ATF-6 (1:1000, Abcam), PPAR-α (1:1000, Santa Cruz Biotechnology), PPAR-β (1:1000, Santa Cruz Biotechnology), PPAR-γ (1:1000, Santa Cruz Biotechnology), phosphorylated and non-phosphorylated forms of ERK, JNK, p38 (1:2000, Santa Cruz Biotechnology), AKT (1:2000, Cell Signaling), and β-tubulin (1:2000, Promega). After the membranes were washed again with PBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using enhanced chemiluminescence western blotting reagents (Amersham Pharmacia Biotech).

2.6. Caspase activity assay

Caspase activity assay was carried out using a fluorometric protease assay kit following the instructions provided by the manufacturer (BioVision). In brief, cells were homogenized on ice with kit-provided lysis buffer. An aliquot of 50 μl of supernatants was incubated with an equal volume of the reaction buffer containing fluorogenic peptide substrate at 37 °C for 1–2 h. Enzymatic release of free fluorogenic moiety was measured by a fluorometer.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. For comparisons, the statistical significance between means was determined using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Indomethacin reduced cell viability in 786-O cells

The expression of COX-2 in 786-O cells was successfully detected by western blotting and immunocytochemistry (data not shown). Initially, we examined the effect of indomethacin, a non-selective COX inhibitor, on the viability of 786-O cells by measuring the reduction of MTS. After 24 h of exposure, indomethacin began to decrease cell viability significantly when the concentration was increased to 100 μM (Fig. 1A). On the other hand, 200 μM of indomethacin remarkably reduced cell viability at 16 h after exposure and the viability continuously

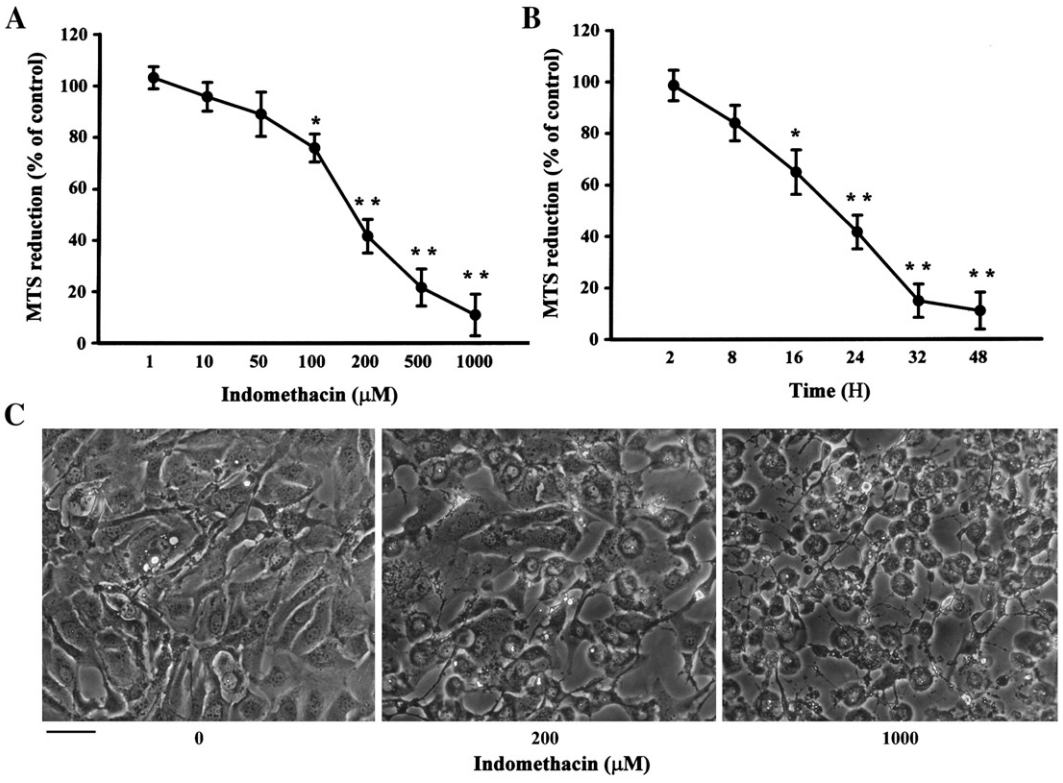

Fig. 1. Effects of indomethacin on cell growth. 786-O cells were treated with various concentrations of indomethacin for 24 h (A) or 786-O cells were treated with 200 μM indomethacin over time (B). Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each control. *$P<0.05$, **$P<0.01$ vs. control, $n=5$. Representative images of phase contrast were obtained 24 h after treatment (C). Scale bar = 60 μm.

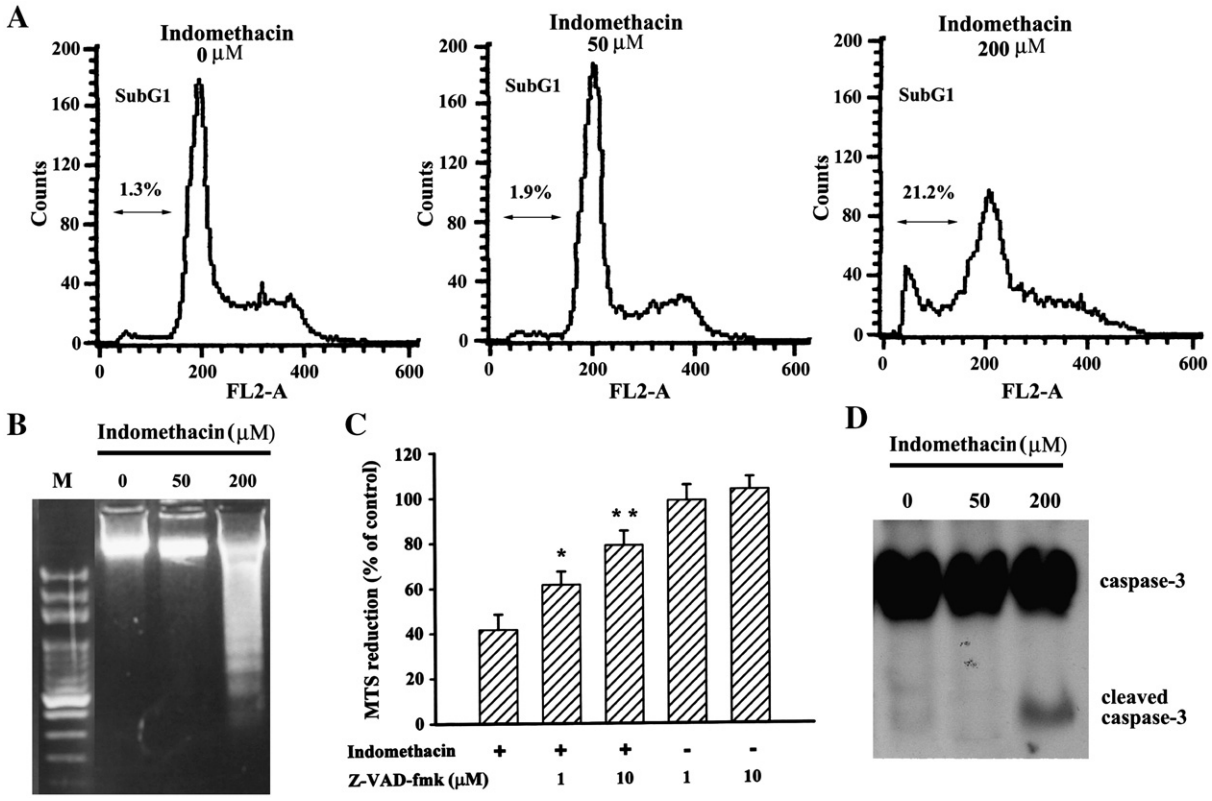

Fig. 2. Effects of indomethacin on cell apoptosis. 786-O cells were treated with various concentrations of indomethacin for 16 h. The cells were harvested and processed by flow-cytometric analysis. FL2A represents the intensity of propidium iodide (A). Cells were harvested and processed to assess DNA fragmentation (B). Protein extracts were isolated and subjected to western blot analysis with antibody against caspase-3 (D). 786-O cells were treated with 200 μM indomethacin, Z-VAD-FMK (1 and 10 μM), or in combination for 24 h. Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each control. *$P<0.05$, **$P<0.01$ vs. indomethacin alone, $n=5$.

decreased over time (Fig. 1B). Furthermore, cell morphology was observed with a light microscope after treatment with indomethacin. The 786-O cells treated with indomethacin showed distinctive morphological changes, including cellular rounding, shrinkage, and membrane blebbing, and separation from neighboring cells (Fig. 1C). The findings indicate that indomethacin concentration- and time-dependently reduced cell viability in 786-O cells.

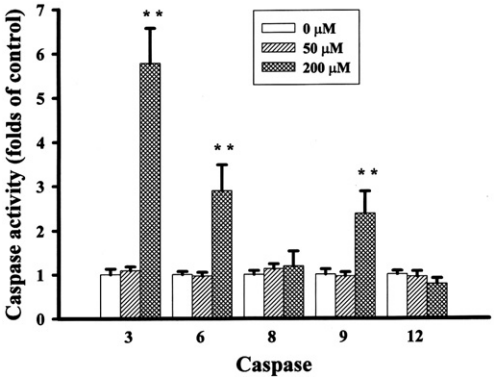

Fig. 3. Effects of indomethacin on caspase activity. 786-O cells were treated with various concentrations of indomethacin for 16 h. Protein extracts were isolated and subjected to fluorogenic protease assay. The assayed caspases included caspase-3, caspase-6, caspase-8, caspase-9, and caspase-12. The activity of each control was defined as 1. **$P<0.01$ vs. each control, $n=5$.

3.2. Indomethacin-induced apoptosis in 786-O cells

To understand the mechanism by which indomethacin caused viability loss in 786-O cells, several experiments were carried out involving apoptosis. First, the occurrence of cell apoptosis was analyzed by flow cytometry. Control cells showed normal distribution in fluorescent peaks. Exposure to indomethacin disrupted the normal distribution and generated a prominent new peak, representing the subG1 peak. The percentage of this subG1 peak in controlled and non-toxic (50 μM of indomethacin) cells was 1.3±0.8% and 1.9±0.6%, respectively, and shifted to 21.2±4.5% in damaged cells (200 μM of indomethacin) (Fig. 2A). DNAs extracted from control and non-damaged (50 μM of indomethacin) cells were found to be intact whereas, in injured cells (200 μM of indomethacin), a characteristic internucleosomal ladder appeared (Fig. 2B). Pharmacological results revealed that indomethacin-induced cytotoxicity was remarkably reduced by a non-selective caspase inhibitor Z-VAD-FMK (Fig. 2C). The proteolytic cleavage and generation of the cleaved product of caspase-3 was found in indomethacin-induced apoptotic cells (Fig. 2D). To further elucidate whether caspase family proteases

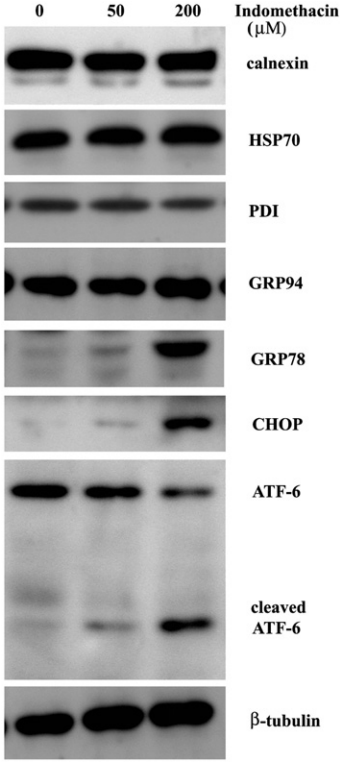

Fig. 4. Effects of indomethacin on ER-related proteins expression. 786-O cells were treated with various concentrations of indomethacin for 16 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against calnexin, HSP70, PDI, GRP94, GRP78, CHOP, ATF-6, and β-tubulin. One of four independent experiments is shown.

are activated in the indomethacin-induced apoptotic process, their proteolytic activities were measured. Elevated caspase-3, caspase-6, and caspase-9 activities were detected in indomethacin-treated cells. However, in the same treated cells, changes in caspase-8 and caspase-12 activity were not found (Fig. 3). Taken together, our findings indicate that the viability loss of 786-O cells caused by indomethacin was associated with caspase-related apoptotic injury.

3.3. Indomethacin-induced endoplasmic reticulum stress in 786-O cells

Endoplasmic reticulum is an interesting organelle which plays diverse roles either in the initiation or inhibition of apoptotic processes (Tsutsumi et al., 2004; Yamazaki et al., 2006). Generally, cells respond to endoplasmic reticulum stress by increasing the expression of genes encoding endoplasmic reticulum molecular chaperones and activating endoplasmic reticulum-related signaling molecules. To gain further insight into the potential involvement of endoplasmic reticulum stress in indomethacin-induced apoptosis, we analyzed the expression of several molecular chaperones and pro-apoptotic transcription factors. Western blotting revealed that the expressions of calnexin, HSP70, PDI, and GRP94 were not changed by indomethacin (Fig. 4). Remarkably, indomethacin-induced elevated expression of the chaperone protein GRP78 and pro-apoptotic protein CHOP (Fig. 4). In addition, in controlled cells, an endoplasmic reticulum transmembrane form of sensing protein ATF-6 was detected, but the processed and active form was not detected. Indomethacin treatment decreased the accumulation of the transmembrane form of ATF-6, but generated the active form, indicating the activation of ATF-6 (Fig. 4). These results indicate that indomethacin treatment initiated endoplasmic reticulum stress involving the induction of a chaperone protein and pro-apoptotic proteins.

3.4. Indomethacin-induced PPAR expression in 786-O cells

PPARs function as critical regulators of diverse cellular processes including cell growth, differentiation, and inflammation (Schoonjans et al., 1997). Increasing evidence has also revealed their involvement in cell apoptosis (Lehmann et al., 1997; Wick et al., 2002). To explore whether activation of PPARs is involved in the mechanism by which indomethacin induces apoptosis, pharmacologically active agents of PPARs

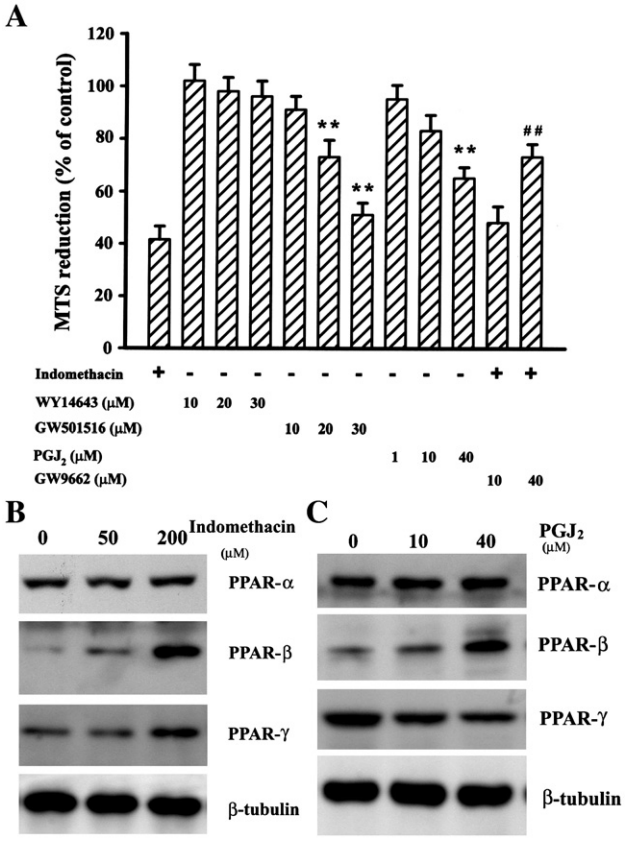

Fig. 5. Roles of PPARs in indomethacin-induced cytotoxicity. 786-O cells were treated with 200 μM indomethacin, WY14643, GW501516, or PGJ_2, or 200 μM indomethacin plus GW9662 for 24 h. Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each control. $^{**}P<0.01$ vs. control and $^{\#\#}P<0.01$ vs. indomethacin alone, $n=5$ (A). 786-O cells were treated with various concentrations of indomethacin for 16 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against PPAR-α, PPAR-β, PPAR-γ and β-tubulin. One of four independent experiments is shown (B). 786-O cells were treated with various concentrations of PGJ_2 for 16 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against PPAR-α, PPAR-β, PPAR-γ and β-tubulin. One of three independent experiments is shown (C).

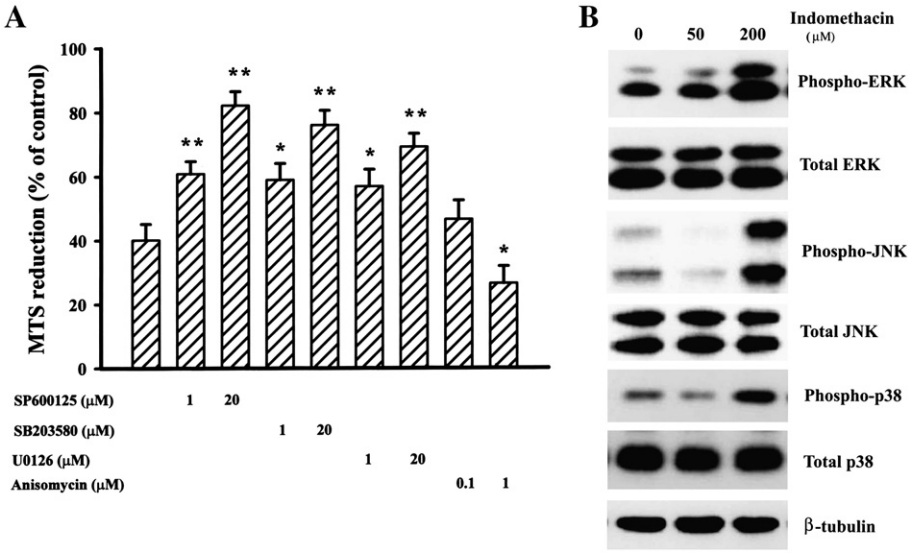

Fig. 6. Roles of MAPKs in indomethacin-induced cytotoxicity. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with SP600125, SB203580, U0126, or anisomycin for 24 h. Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each control. *$P<0.05$ and **$P<0.01$ vs. indomethacin alone, $n=5$ (A). 786-O cells were treated with various concentrations of indomethacin for 2 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against phospho- and total-ERK, JNK, and p38, and β-tubulin. One of four independent experiments is shown (B).

were used. Incubation with the synthetic PPAR-α agonist, [[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio] acetic acid (WY14643, Tocris) (Issemann and Green, 1990), did not affect 786-O cell viability. In contrast, when incubated with a selective PPAR-β/δ agonist, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid (GW501516, Calbiochem) (Oliver et al., 2001), or the natural PPAR-γ agonist, 15-deoxy-delta 12,14-prostaglandin J2 (PGJ_2) (Forman et al., 1995), a decrease in 786-O cell viability was observed. 2-Chloro-5-nitro-*N*-phenyl-benzamide (GW9662, Sigma), a selective and irreversible PPAR-γ antagonist (Clay et al., 2002), partially protected 786-O cells from indomethacin-induced cell death (Fig. 5A). To further investigate the contribution of PPARs to indomethacin-induced loss in viability, the expression of PPAR proteins was assessed. The expression of PPAR-α in 786-O cells was hardly affected by indomethacin. In contrast, a higher concentration of indomethacin treatment upregulated the protein expression of PPAR-β and PPAR-γ by 6.7±1.9 fold and 2.4±0.9 fold, respectively (Fig. 5B). The effect of PGJ_2 on PPARs protein expression was unexpected. PGJ_2 treatment increased PPAR-β expression (3.8±1.1 fold) but slightly reduced PPAR-γ expression (0.64±0.09 fold). The expression of PPAR-α was not changed by PGJ_2 (Fig. 5C). These findings indicate that PPARs, especially PPAR-β and PPAR-γ, might be key determinants in indomethacin-induced 786-O cell death.

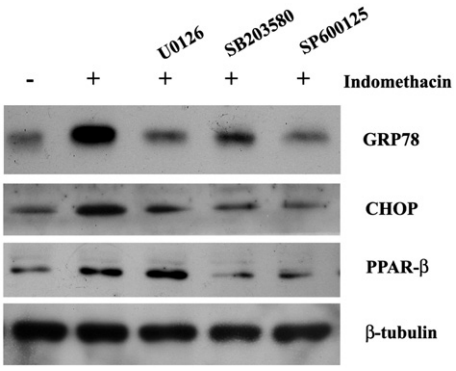

Fig. 7. Roles of MAPKs in indomethacin-induced gene induction. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with U0126 (20 μM), SB203580 (20 μM), or SP600125 (20 μM) for 16 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against GRP78, CHOP, PPAR-β, and β-tubulin. One of four independent experiments is shown.

3.5. Indomethacin-induced MAPK activation in 786-O cells

To examine whether MAPKs play roles in indomethacin-induced 786-O cell death, we determined cytotoxicity in the presence of MAPK inhibitors and activators. Treatment of 786-O cells with 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126, Tocris) (an inhibitor of ERK); 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl] pyridine (SB203580, Tocris) (an inhibitor of p38 MAPK); or anthra(1,9-cd)pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone (SP600125, Tocris) (an inhibitor of JNK), led to a decrease in indomethacin-induced viability loss (Fig. 6A). In contrast, anisomycin, an activator of MAPKs (Chang et al., 2005), exacerbated indomethacin-induced cell death (Fig. 6A). Pharmacological findings have revealed that the suppression of MAPK activities including ERK, p38 MAPK, and JNK protects cells against indomethacin-induced toxicity, indicating the activation of MAPKs by indomethacin. To parallel these observations, the changes in MAPK phosphorylation were determined by western blotting to correlate their activities. As shown in Fig. 6B, indomethacin concentration-dependently induced an increase in ERK, p38 MAPK, and JNK phosphorylation.

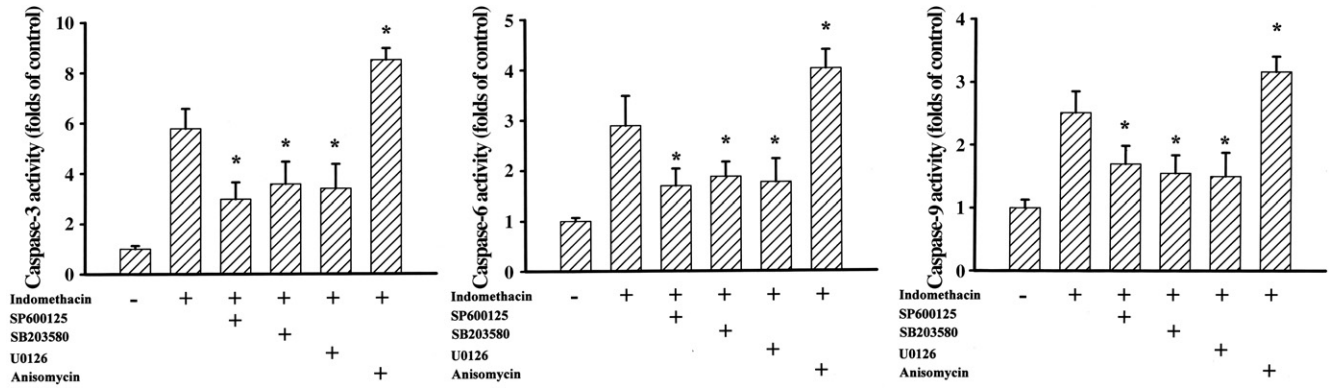

Fig. 8. Roles of MAPKs in indomethacin-induced caspase activity. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with U0126 (20 μM), SB203580 (20 μM), SP600125 (20 μM), or anisomycin (1 μM) for 16 h. Protein extracts were isolated and subjected to fluorogenic protease assay. The assayed caspases included caspase-3, caspase-6, and caspase-9. The activity of each control was defined as 1. *$P<0.05$ vs. each indomethacin control, $n=5$.

Our experiments using specific MAPK inhibitors suggest that the activation of MAPKs is critical to indomethacin-induced 786-O cell death (Fig. 6A). To delineate the potential downstream effectors of MAPKs that mediate indomethacin-induced cell death, we next sought to verify whether the induction of GRP78, CHOP, and PPAR-β by indomethacin was

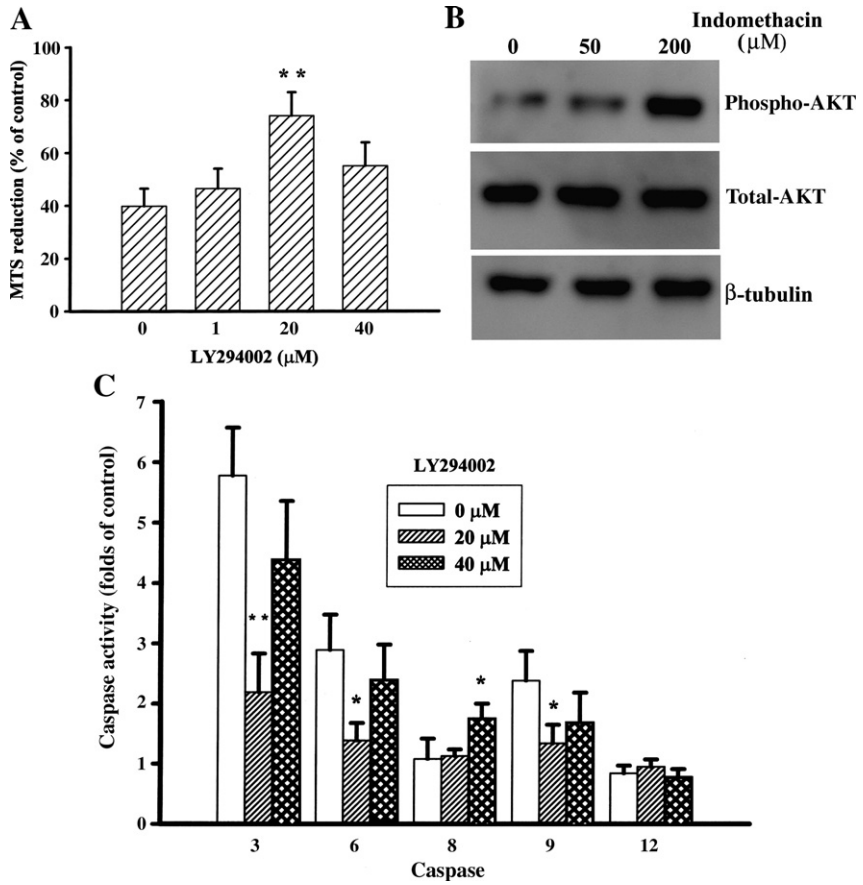

Fig. 9. Effects of AKT on indomethacin-induced alterations. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with various concentrations of LY294002 for 24 h. Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each non-treated control. **$P<0.01$ vs. indomethacin alone, $n=5$ (A). 786-O cells were treated with various concentrations of indomethacin for 2 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against phospho- and total-AKT, and β-tubulin. One of four independent experiments is shown (B). 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with various concentrations of LY294002 for 16 h. Protein extracts were isolated and subjected to fluorogenic protease assay. The assayed caspases included caspase-3, caspase-6, caspase-8, caspase-9, and caspase-12. The activity of each control (0 μM of indomethacin) was defined as 1. *$P<0.05$ and **$P<0.01$ vs. each indomethacin control alone, $n=5$ (C).

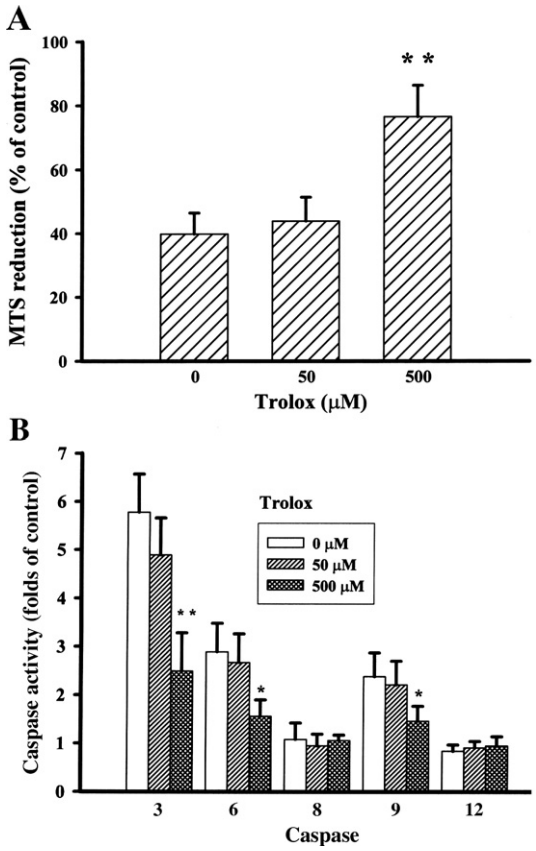

Fig. 10. Effects of Trolox on indomethacin-induced alterations. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with various concentrations of Trolox for 24 h. Cell viability was determined by MTS reduction assay. Values are expressed as a percentage of each non-treated control. $^{**}P<0.01$ vs. indomethacin alone, $n=5$ (A). 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with various concentrations of Trolox for 16 h. Protein extracts were isolated and subjected to fluorogenic protease assay. The assayed caspases included caspase-3, caspase-6, caspase-8, caspase-9, and caspase-12. The activity of each control (0 μM of indomethacin) was defined as 1. $^{*}P<0.05$ and $^{**}P<0.01$ vs. each indomethacin control alone, $n=5$ (B).

under the control of MAPKs. Western blotting revealed that indomethacin-induced GRP78 protein expression was attenuated by U0126 (24±5.6% of the indomethacin control), SB203580 (37±6.6% of the indomethacin control), and SP600125 (19±5.9% of the indomethacin control) (Fig. 7). Similar suppression of CHOP protein expression was also apparent in U0126 (41±4.6% of the indomethacin control), SB203580 (38±5.6% of the indomethacin control), and SP600125 (34±7.1% of the indomethacin control) (Fig. 7). Regarding the expression of PPAR-β, SB203580 (18±6.6% of the indomethacin control) and SP600125 (21±6.1% of the indomethacin control) ameliorated its induction, whereas indomethacin-induced PPAR-β protein expression was not affected by U0126 (98±7.1% of the indomethacin control) (Fig. 7). We also delineated the correlation between MAPK and indomethacin-induced caspase activation. Inactivation of ERK, p38 MAPK, and JNK by pharmacological inhibitors suppressed indomethacin-induced caspase activities. On the other hand, anisomycin further increased indomethacin-induced caspase activation (Fig. 8). These findings indicate that MAPKs might sense and amplify intracellular signaling of indomethacin through similar or distinct downstream effectors.

3.6. Indomethacin-induced AKT activation in 786-O cells

To elicit the effect of phosphoinositide-3 kinase (PI3K)/AKT in indomethacin-induced cytotoxicity, pharmacological inhibitor was used. Twenty micromolars of 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride (LY294002, Tocris), a PI3K/AKT inhibitor, attenuated indomethacin-induced cytotoxicity, whereas no cytoprotective effect was found at a higher concentration of LY294002 (Fig. 9A). Western blotting revealed that indomethacin increased AKT phosphorylation (Fig. 9B). Cytoprotective concentration of LY294002 also decreased indomethacin-induced caspase-3, caspase-6, and caspase-9 activities. In contrast, a non-protective concentration of LY294002 was shown to have no effect on these caspase activities. Interestingly, a higher concentration of LY294002 slightly increased caspase-8 activity (Fig. 9C). These findings indicate that the effect of LY294002 on indomethacin-induced 786-O cell changes varies with the concentration.

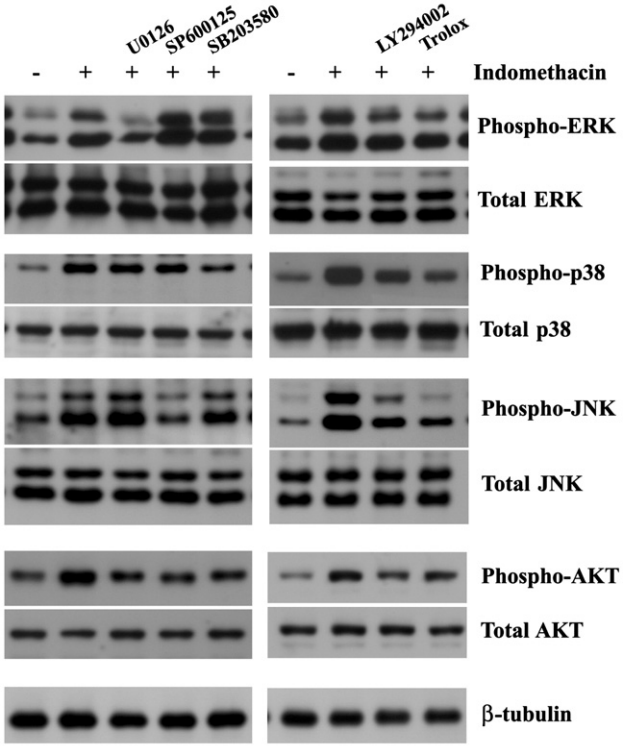

Fig. 11. Effects of pharmacological inhibitors on indomethacin-induced alterations. 786-O cells were treated with medium or 200 μM indomethacin alone, or 200 μM indomethacin in combination with U0126 (20 μM), SB203580 (20 μM), SP600125 (20 μM), LY294002 (20 μM), or Trolox (500 μM) for 2 h. Protein extracts were isolated and subjected to western blot analysis with antibodies against phospho- and total-ERK, p38, JNK, and AKT, and β-tubulin. One of four independent experiments is shown.

3.7. Antioxidants reduced indomethacin-induced cytotoxicity

It has been reported that reactive oxygen species are involved in the initiation of apoptosis (Kusuhara et al., 1999). In this study, we found that the antioxidant 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Tocris) suppressed indomethacin-induced cytotoxicity (Fig. 10A) and caspase activities (Fig. 10B), indicating the involvement of reactive oxygen species in indomethacin-induced apoptosis. The abovementioned findings suggest that indomethacin might induce ERK, p38 MAPK, JNK, and AKT phosphorylation leading to the initiation of apoptosis. A cytoprotective concentration of Trolox significantly reduced indomethacin-induced increased phosphorylation in ERK (29±5.4% of the indomethacin control), p38 MAPK (24±6.6% of the indomethacin control), JNK (21±6.0% of the indomethacin control), and AKT (31±6.9% of the indomethacin control) (Fig. 11). These findings suggest that reactive oxygen species might lie upstream from these signaling molecules to transduce indomethacin's signals. Western blotting data further revealed that inhibition of PI3K/AKT attenuated indomethacin-induced increased phosphorylation in ERK (39±6.3% of the indomethacin control), p38 MAPK (31±6.2% of the indomethacin control), and JNK (29±7.3% of the indomethacin control) (Fig. 11). On the other hand, increased phosphorylation of AKT by indomethacin was downregulated in the presence of U0126 (41±5.1% of the indomethacin control), SP600125 (32±6.9% of the indomethacin control), or SB203580 (43±5.6% of the indomethacin control) (Fig. 11). Taken together, the results suggest that a mutual interaction between MAPKs and AKT exists in the transmission of signals following indomethacin treatment in 786-O cells.

4. Discussion

In this study, we demonstrated that indomethacin-induced cell apoptosis in 786-O cells. However, the selective COX-2 inhibitor NS-398 failed to induce apoptosis (data not shown), suggesting that additional targets distinct from COX mediate indomethacin induction of apoptosis in 786-O cells. Our data suggest that the signaling molecules including reactive oxygen species, the MAPK family and AKT are important regulators of cell viability in 786-O cells and may be a possible COX-independent target for indomethacin. Cellular molecules such as GRP78, CHOP, and PPAR-β are potential downstream effectors of MAPKs in mediating indomethacin-transduced signals in cell survival.

Cells respond to endoplasmic reticulum stress by activating a self-protective mechanism, involving induction of molecular chaperones and initiating downstream signaling processes to survive endoplasmic reticulum stress conditions. If these adaptive responses are not sufficient to relieve cells from endoplasmic reticulum stress, cells undergo apoptosis to destroy the endoplasmic reticulum stress-damaged cells themselves (Momoi, 2004; Shen et al., 2004). In this study, the cleavage and activation of ATF-6 and induced expression of CHOP were found in indomethacin-treated cells (Fig. 4). Although the mechanism is still unclear, CHOP has been shown to be involved in the regulation of apoptosis associated with endoplasmic reticulum stress (Zinszner et al., 1998; Yoshida et al., 2000). Thus, a CHOP-dependent mechanism might play a role in indomethacin-induced 786-O cell apoptosis. Another family of endoplasmic reticulum stress sensing molecules is molecular chaperone proteins. Among the analyzed chaperones, an increase in GRP78, a characteristic chaperone of the endoplasmic reticulum, was detected (Fig. 4). As an endoplasmic reticulum-resident molecular chaperone and the final product of the unfolded protein response (UPR), GRP78 is involved in the folding of unfolded proteins and also acts as an apoptotic regulator. Evidence shows that GRP78 might prevent endoplasmic reticulum stress-induced cell death, partly through caspase-12 sequestration, an endoplasmic reticulum stress-related apoptotic caspase (Nakagawa et al., 2000). In our study, the caspase-12 activity remained unchanged in treated cells (Fig. 3), implying the adaptive and protective effects of induced GRP78. Taken together, our findings indicate the importance and involvement of the endoplasmic reticulum organelle in indomethacin-induced 786-O cell apoptosis.

In searching for further cytotoxic characteristics, we found that indomethacin increased protein expression markedly in PPAR-β and slightly in PPAR-γ, but was ineffective in PPAR-α (Fig. 5B). The PPAR family comprises three closely related gene products, PPAR-α, PPAR-β/δ, and PPAR-γ (Schoonjans et al., 1997). Evidence suggests that, in addition to their physiological functions, activation of PPAR-α provides an anti-apoptotic mechanism (Roberts et al., 1998) but activation of PPAR-γ promotes apoptosis (Lehmann et al., 1997; Wick et al., 2002). In contrast to this, although PPAR-β/δ is ubiquitously expressed, its functional significance is currently unknown (Lemberger et al., 1996). Correlating the induction of PPAR-β/δ and PPAR-γ with indomethacin-induced cytotoxicity, the results of pharmacologically active compounds were in accordance with the conclusion that both induced PPAR-β/δ and PPAR-γ were strongly associated with cytotoxicity in 786-O cells (Fig. 5A). Unexpectedly, PGJ_2 stimulation slightly reduced endogenous PPAR-γ expression but significantly induced PPAR-β expression (Fig. 5C). The coincident induction of PPAR-β was paralleled with the onset of PGJ_2-induced 786-O cell cytotoxicity (Fig. 5A and C). This regulation of PPAR expression by PGJ_2 was also documented in HT-29 colon cancer cells (Chen and Tseng, 2005). Our findings together with those of other reports reveal the potential contribution of PPAR-β in apoptosis.

MAPKs can be activated by a variety of intrinsic and extrinsic stimuli and recent data suggests that they may mediate apoptotic signaling induced by anti-neoplastic agents (Fan and Chambers, 2001). Our results show that indomethacin-induced activation of MAPKs (Fig. 6B). The experiments using specific inhibitors or activators for MAPKs strongly suggest that activation of ERK, p38 MAPK, and JNK were critical in mediating indomethacin-induced apoptotic changes (Figs. 6A and 8). Activation of ERK is generally related to growth stimulating actions of many growth factors. However, recent research indicates that growth inhibition can also result from ERK activation (Tsukada et al., 2001; Lahlou et al., 2003).

Accordingly, MAPKs might function in either a pro-apoptotic or anti-apoptotic capacity. Signal transduction pathways convey signals into the cell nucleus in order to initiate a program of gene expression that is characteristic for particular stimuli. Related studies and our results demonstrate that GRP78, CHOP, and PPAR-β are among the candidate genes (Zinszner et al., 1998; Yoshida et al., 2000). Evidence has revealed that the activation of MAPKs is involved in the rapid induction of GRP78 and CHOP (Wang and Ron, 1996; Chen et al., 1998). In this study, the increased expression of GRP78 and CHOP after indomethacin treatment was significantly suppressed by U0126, SB203580, and SP600125 (Fig. 7), indicating that the activation of ERK, p38 MAPK, and JNK controlled their expression in indomethacin-treated 786-O cells. Similar regulatory pathways showed that the induction of PPAR-β was under the control of p38 MAPK and JNK. In contrast to this, the induction of PPAR-β was independent of the activation of ERK (Fig. 7). Taken together, these results demonstrate that cell type, state of activation, and cellular context might be key determinants in the regulation of apoptosis by MAPKs.

In analyzing AKT, similar pharmacological protection, protein phosphorylation, and caspase activity downregulation were observed. However, the protective effect of inhibitors was lost when the concentration was increased (Fig. 9). Evidence suggests that inhibition of caspase-8 activation is one of the anti-apoptotic effects of AKT (Panka et al., 2001). In this study, we detected an elevation of caspase-8 activity in inhibitor-treated non-protective cells (Fig. 9C), indicating an association between high AKT phosphorylation and low caspase-8 activity. Significantly, a protective concentration of LY294002 attenuated indomethacin-induced increased phosphorylation of ERK, p38 MAPK, and JNK (Fig. 11). On the other hand, individual inhibitors of ERK, p38 MAPK, and JNK also reduced indomethacin-induced increased phosphorylation of AKT (Fig. 11). Therefore, the outcome of elevated AKT in indomethacin-treated 786-O cells can be separated into two consequences: induction of MAPK phosphorylation leading to cytotoxicity and inhibition of caspase-8 activation to restrain apoptosis. Mutual amplification between MAPKs and AKT might be the key determinant of indomethacin-induced apoptosis.

We found that the antioxidant Trolox remarkably suppressed increased phosphorylation of ERK, p38 MAPK, JNK, and AKT (Fig. 11), effectively decreased caspase activation (Fig. 10B), and protected the cells from death (Fig. 10A), indicating the involvement of reactive oxygen species in indomethacin-induced apoptosis. That is, indomethacin-induced apoptosis appears to be closely associated with oxidative stress. In our experiments, the specific sources of reactive oxygen species generation were not characterized. Evidence indicates that an alternate mechanism of action of NSAIDs involves inhibition of oxidative phosphorylation and disruption of mitochondrial energy metabolism (Mahmud et al., 1996; Jacob et al., 2001). This uncoupling of mitochondrial respiration might lead to the generation of reactive oxygen species. However, whether the uncoupling of oxidative phosphorylation is responsible for indomethacin-induced oxidative stress and apoptosis in 786-O cells remains further investigation.

In this study, the potential contribution of PTEN, Bcl-2, p53, and cell cycle proteins was not addressed. Instead, we focused on the elucidation of related signaling molecules in response to indomethacin treatment in 786-O cells. In conclusion, based on pharmacological and biochemical studies, we provided evidence of a possible pathway of apoptosis induced by indomethacin in 786-O cells. The generation of reactive oxygen species is a crucial event and lies upstream from the signaling molecules including ERK, p38 MAPK, JNK, and AKT. Mutual activation occurs between AKT and MAPKs. Cytotoxicity is associated closely with CHOP and PPAR-β expression and caspase activation. Both gene induction and caspase activation are directly or indirectly under the control of MAPKs. These findings establish a mechanistic link between the reactive oxygen species, MAPK pathway, AKT pathway, and indomethacin-induced cellular alterations and apoptosis. It should be noted that the concentration of indomethacin required to decrease cell viability and induce apoptosis greatly exceeded the clinically therapeutic concentration (<10 μM). Therefore, the therapeutic implication of indomethacin in chemoprevention should be paid more concerns and merits further investigation.

Acknowledgment

This study was supported by a grant from Taichung Veterans General Hospital (TCVGH-945004B), Taiwan.

References

Bakhle, Y.S., 2001. COX-2 and cancer: a new approach to an old problem. Br. J. Pharmacol. 134, 1137–1150.

Carvalho, H., Evelson, P., Sigaud, S., Gonzalez-Flecha, B., 2004. Mitogen-activated protein kinases modulate H_2O_2-induced apoptosis in primary rat alveolar epithelial cells. J. Cell Biochem. 92, 502–513.

Chang, C.C., Ou, Y.C., Raung, S.L., Chen, C.J., 2005. Antiviral effect of dehydroepiandrosterone on Japanese encephalitis virus infection. J. Gen. Virol. 86, 2513–2523.

Chen, C.J., Liao, S.L., 2002. Oxidative stress involved in astrocytic alterations induced by manganese. Exp. Neurol. 175, 216–225.

Chen, Z.Y., Tseng, C.C., 2005. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 up-regulates Krüppel-like factor 4 expression independently of peroxisome proliferator-activated receptor γ by activating the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signal transduction pathway in HT-29 colon cancer cells. Mol. Pharmacol. 68, 1203–1213.

Chen, K.D., Chen, L.Y., Huang, H.L., Lieu, C.H., Chang, Y.N., Chang, M.D., Lai, Y.K., 1998. Involvement of p38 mitogen-activated protein kinase signaling pathway in the rapid induction of the 78-kDa glucose-regulated protein in 9L rat brain tumor cells. J. Biol. Chem. 273, 749–755.

Chen, Q., Shinohara, N., Abe, T., Watanabe, T., Nonomura, K., Koyanagi, T., 2004. Significance of COX-2 expression in human renal cell carcinoma cell lines. Int. J. Cancer 108, 825–832.

Chu, E.C., Chai, J., Tarnawski, A.S., 2004. NSAIDs activate PTEN and other phosphatases in human colon cancer cells: novel mechanism for chemopreventive action of NSAIDs. Biochem. Biophys. Res. Commun. 320, 875–879.

Clay, C.E., Monjazeb, A., Thorburn, J., Chilton, F.H., 2002. High KP, 15-Deoxy-delta12,14-prostaglandin J2-induced apoptosis does not require PPARgamma in breast cancer cells. J. Lipid Res. 43, 1818–1828.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E., 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231–241.

Dubois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., Van De Putte, L.B., Lipsky, P.E., 1998. Cyclooxygenase in biology and disease. FASEB J. 12, 1063–1073.

Duperron, C., Castonguay, A., 1997. Chemopreventive efficacies of aspirin and sulindac against lung tumorigenesis in A/J mice. Carcinogenesis 18, 1001–1006.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S., DuBois, R.N., 1994. Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107, 1183–1188.

Fan, M., Chambers, T.C., 2001. Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist. Updat. 5, 253–267.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M., 1995. 15-Deoxy-delta12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83, 803–812.

Fosslien, E., 2000. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Crit. Rev. Clin. Lab. Sci. 37, 431–502.

Garrington, T.P., Johnson, G.L., 1999. Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr. Opin. Cell Biol. 11, 211–218.

Hanif, R., Pittas, A., Feng, Y., Koutsos, M.I., Qiao, L., Staiano-Coico, L., Shiff, S.I., Rigas, B., 1996. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by prostaglandin-independent pathway. Biochem. Pharmacol. 52, 237–245.

Ho, C.C., Yang, X.W., Lee, T.L., Liao, P.H., Yang, S.H., Tsai, C.H., Chou, M.Y., 2003. Activation of p53 signalling in acetylsalicylic acid-induced apoptosis in OC2 human oral cancer cells. Eur. J. Clin. Invest. 33, 875–882.

Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347, 645–650.

Jacob, M., Bjarnason, I., Rafi, S., Wrigglesworth, J., Simpson, R.J., 2001. A study of the effects of indomethacin on liver mitochondria from rats, mice and humans. Aliment. Pharmacol. Ther. 15, 1837–1842.

Kern, M.A., Haugg, A.M., Koch, A.F., Schilling, T., Breuhahn, K., Walczak, H., Fleischer, B., Trautwein, C., Michalski, C., Schulze-Bergkamen, H., Friess, H., Stremmel, W., Krammer, P.H., Schirmacher, P., Muller, M., 2006. Cyclooxygenase-2 inhibition induces apoptosis signaling via death receptors and mitochondria in hepatocellular carcinoma. Cancer Res. 66, 7059–7066.

Khwaja, F., Allen, J., Lynch, J., Andrews, P., Djakiew, D., 2004. Ibuprofen inhibits survival of bladder cancer cells by induced expression of the $p75^{NTR}$ tumor suppressor protein. Cancer Res. 64, 6207–6213.

Kusuhara, H., Komatsu, H., Sumichika, H., Sugahara, K., 1999. Reactive oxygen species are involved in the apoptosis induced by nonsteroidal anti-inflammatory drugs in cultured gastric cells. Eur. J. Pharmacol. 383, 331–337.

Lahlou, H., Saint-Laurent, N., Esteve, J.P., Eychene, A., Pradayrol, L., Pyronnet, S., Susini, C., 2003. sst2 Somatostatin receptor inhibits cell proliferation through Ras-, Rap1- and B-Raf-dependent ERK2 activation. J. Biol. Chem. 278, 39356–39371.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptor α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406–3410.

Lemberger, T., Desvergne, B., Wahli, W., 1996. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu. Rev. Cell Dev. Biol. 12, 335–363.

Liu, C.H., Chang, S.H., Narko, K., Trifan, O.C., Wu, M.T., Smith, E., Haudenschild, C., Lane, T.F., Hla, T., 2001. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J. Biol. Chem. 276, 18563–18569.

Lu, S., Yu, G., Zhu, Y., Archer, M.C., 2005. Cyclooxygenase-2 overexpression in MCF-10F human breast epithelial cells inhibits proliferation, apoptosis and differentiation, and causes partial transformation. Int. J. Cancer 116, 847–852.

Mahmud, T., Rafi, S.S., Scott, D.L., Wrigglesworth, J.M., Bjarnason, I., 1996. Nonsteroidal anti-inflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation. Arthritis Rheum. 39, 1998–2003.

Marnett, L.J., 1992. Aspirin and the potential role of prostaglandins in colon cancer. Cancer Res. 52, 5575–5589.

Martin, S., Phillips, D.C., Szekely-Szucs, K., Elghazi, L., Desmots, F., Houghton, J.A., 2005. Cyclooxygenase-2 inhibition sensitizes human colon carcinoma cells to TRAIL-induced apoptosis through clustering of DR5 and concentrating death-inducing signaling complex components into ceramide-enriched caveolae. Cancer Res. 65, 11447–11458.

Miyata, Y., Koga, S., Kanda, S., Nishikido, M., Hayashi, T., Kanetake, H., 2003. Expression of cyclooxygenase-2 in renal cell carcinoma: correlation with tumor cell proliferation, apoptosis, angiogenesis, expression of matrix metalloproteinase-2, and survival. Clin. Cancer Res. 9, 1741–1749.

Momoi, T., 2004. Caspases involved in ER stress-mediated cell death. J. Chem. Neuroanat. 28, 101–105.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J., 2000. Caspase-12 mediates endoplasmic reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403, 98–103.

Oliver Jr., W.R., Shenk, J.L., Snaith, M.R., Russell, C.S., Plunket, K.D., Bodkin, N.L., Lewis, M.C., Winegar, D.A., Sznaidman, M.L., Lambert, M.H., Xu, H.E., Sternbach, D.D., Kliewer, S.A., Hansen, B.C., Willson, T.M., 2001. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc. Natl. Acad. Sci. U. S. A. 98, 5306–5311.

Panka, D.J., Mano, T., Suhara, T., Walsh, K., Mier, J.W., 2001. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J. Biol. Chem. 276, 6893–6896.

Roberts, R.A., James, N.H., Woodyatt, N.J., Macdonald, N., Tugwood, J.D., 1998. Evidence for the suppression of apoptosis by the peroxisome proliferators-activated receptor α (PPAR α). Carcinogenesis 19, 43–48.

Saito, S., Takahashi, S., Takagaki, N., Hirose, T., Sakai, T., 2003. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induces apoptosis through activation of the CHOP gene in HeLa cells. Biochem. Biophys. Res. Commun. 311, 17–23.

Schoonjans, K., Martin, G., Staels, B., Auwerx, J., 1997. Peroxisome proliferator-activated receptors: orphans with ligands and functions. Curr. Opin. Lipidol. 8, 159–166.

Shen, X., Zhang, K., Kaufman, R.J., 2004. The unfolded protein response—a stress signaling pathway of the endoplasmic reticulum. J. Chem. Neuroanat. 28, 79–92.

Shi, D.Y., Deng, Y.R., Liu, S.L., Zhang, Y.D., Wei, L., 2003. Redox stress regulates cell proliferation and apoptosis of human hepatoma through Akt protein phosphorylation. FEBS Lett. 542, 60–64.

Souza, R.F., Shewmake, K., Beer, D.G., Cruer, B., Spechler, S.J., 2000. Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. Cancer Res. 60, 5767–5772.

Tsukada, Y., Miyazawa, K., Kitamura, N., 2001. High-intensity ERK signal mediates hepatocyte growth factor-induced proliferation inhibition of the human hepatocellular carcinoma cell line HepG2. J. Biol. Chem. 276, 40968–40976.

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

Wang, X.Z., Ron, D., 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase. Science 272, 1347–1349.

Wick, M., Hurteau, G., Dessev, C., Chan, D., Geraci, M.W., Winn, R.A., Heasley, L.E., Nemenoff, R.A., 2002. Peroxisome proliferator-activated receptor-γ is a target of nonsteroidal anti-inflammatory drugs mediating cyclooxygenase-independent inhibition of lung cancer cell growth. Mol. Pharmacol. 62, 1207–1214.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331.

Yamazaki, T., Muramoto, M., Oe, T., Morikawa, N., Okitsu, O., Nagashima, T., Nishimura, S., Katayama, Y., Kita, Y., 2006. Diclofenac, a non-steroidal anti-inflammatory drug, suppresses apoptosis induced by endoplasmic reticulum stresses by inhibiting caspase signaling. Neuropharmacology 50, 558–567.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., Mori, K., 2000. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF)

directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol. Cell. Biol. 20, 6755–6767.

Zhang, G.S., Liu, D.S., Xia, M., 2003. Indomethacin-induced HL-60 leukemic cell apoptosis and the activation of C-*jun* NH_2-terminal kinase signal transduction pathway. Hunan Yi Ke Da Xue Xue Bao 28, 557–562.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L., Ron, D., 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev. 12, 982–995.