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COX-2 regulates E-cadherin expression through the NF-κB/Snail signaling pathway in gastric cancer

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Abstract. Cyclooxygenase-2 (COX-2) participates in cancer invasion and metastasis by decreasing the expression of E-cadherin. However, the molecular mechanisms through which COX-2 regulates E-cadherin expression and function have not yet been fully elucidated. The aim of this study was to investigate the possible molecular mechanisms through which COX-2 regulates E-cadherin expression in gastric cancer. The mRNA and protein expression of COX-2, nuclear factor-κB (NF-κB), Snail and E-cadherin was detected in gastric cancer cells by quantitative PCR and western blot analysis, respectively. The expression of these genes was also detected in healthy gastric mucosa and gastric cancer tissues by immunohistochemistry. We detected various levels of COX-2, nuclear factor-κB (NF-κB), Snail and E-cadherin expression in the normal gastric mucosa and cancer tissues; however, the expression patterns differed: the increased expression of COX-2, NF-κB and Snail was observed in the gastric cancer tissues, whereas there was a considerable reduction in E-cadherin expression in the cancer tissues compared to the normal gastric mucosa. The expression patterns of COX-2, NF-κB and Snail were similar. The increased expression of COX-2 in the gastric cancer tissues closely correlated with the increased expression of NF-κB and Snail, but inversely correlated with the expression of E-cadherin. Treatment of the SGC7901 cells (which express high levels of COX-2) with celecoxib, a COX-2 inhibitor, not only led to a marked dose- and time-dependent decrease in the expression of COX-2, NF-κB and Snail, but also led to a significant increase in the expression of E-cadherin, and this was associated with a reduction in cell invasion. By contrast, the same treatment did not alter the expression of these genes in another gastric cancer cell line, MGC803 (which barely expresses COX-2). These data suggest that COX-2 regulates the expression of E-cadherin through the NF-κB and Snail signaling pathway in gastric cancer.

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Abbreviations: COX-2, cyclooxygenase-2; NF-κB, nuclear factor-κB

Key words: gastric cancer, cyclooxygenase-2, nuclear factor-κB, Snail, E-cadherin

Introduction

Gastric cancer is one of the most common malignant tumors worldwide. It was estimated that in 2008, 998,000 new cases of gastric cancer were diagnosed (representing 7.8% of all new tumor cases) and 736,000 patients died of this malignancy (accounting for 9.7% of all tumor deaths) (1). Invasion and metastasis are major factors for poor prognosis and mortality in gastric cancer. Thus, understanding the molecular mechanisms involved in the invasion and metastasis of gastric cancer is necessary for developing effective treatment options to combat this disease (2).

Cancer invasion and metastasis are generally associated with molecular abnormalities in adhesion molecules. E-cadherin is one of the most important adhesion molecules and is essential for the maintenance of intact cellular functionality. The inhibition of tumor invasion and metastasis are among the most important biological functions of E-cadherin, as its abnormal expression and function have been observed in several epithelium-derived cancers including gastric cancer (2-4).

A number of studies have shown that cyclooxygenase-2 (COX-2) affects many aspects of fundamental cellular processes, such as the promotion of cell proliferation, inhibi-

tion of apoptosis and the enhancement of neovascularization. Thus, COX-2 significantly contributes to carcinogenesis (5,6). Previous studies have indicated that COX-2 participates in cancer invasion and metastasis by decreasing the expression of E-cadherin (7-9). However, the molecular mechanisms through which COX-2 regulates E-cadherin expression and function have not yet been fully elucidated.

In a previous study, we focused on the role of inflammation-related gastric cancer, with particular interest in the role of COX-2 and NF-κB in the development of gastric cancer (10). In this study, we aimed to determine the correlation between COX-2, nuclear factor-κB (NF-κB), Snail and E-cadherin expression in gastric cancer tissues and cells in order to elucidate the possible molecular mechanisms through which COX-2 regulates E-cadherin expression. The data presented in this study may aid in the development of novel therapeutic approaches for combating the invasion and metastasis of gastric cancer.

Materials and methods

Materials. The SGC7901, AGS, BGC823 and MGC803 gastric cancer cell lines were purchased from the Shanghai Institute of Life Sciences of Chinese Academy of Sciences (Shanghai, China). Celecoxib, a selective COX-2 inhibitor, was a gift from the Faculty of Medicine, University of Hong Kong, Hong Kong, China. RPMI-1640 and other cell culture supplements were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). The RNA extraction kit and protein extraction kit were purchased from Shanghai Biological Engineering Materials Co., Ltd. (Shanghai, China). The reverse transcription kit and quantitative PCR (qPCR) kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Polyclonal antibodies for COX-2 and Snail were purchased from Abcam Ltd. (Cambridge, UK). Polyclonal antibodies against NF-κB and E-cadherin, as well as monoclonal antibody against β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Millicell cell culture inserts were obtained from Millipore (Billerica, MA, USA). ECM Gel was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Healthy gastric mucosa, gastric cancer tissues and immunostaining. Paraffin-embedded blocks of healthy human gastric mucosa (n=32) and gastric cancer tissue (n=189) were used to construct tissue microarrays (TMAs). Gastric cancer tissues were obtained from patients with gastric cancer who underwent surgery at the Wuwei Tumor Hospital, Wuwei, China. The healthy gastric mucosal tissues were obtained from individuals who underwent gastric cancer screening endoscopy at the same hospital. The diagnosis of gastric adenocarcinoma was based on the WHO diagnosis criteria, and was confirmed by two independent pathologists. Patient demographics and TMA construction were described in our previous study (10). For immunohistochemical staining, the TMA slides were incubated for 1 h at 37˚C with primary antibody against the following proteins: COX-2 (1:200), NF-κB p65 (1:200), Snail (1:200) and E-cadherin (1:250). The slides were then washed three times with phosphate-buffered saline (PBS), incubated with biotin-conjugated secondary antibody (1:150) for 60 min at 37˚C, washed with PBS and then incubated with strepavidin horseradish peroxidase (SHRP) (Thermo Scientific, USA) for 40 min at room temperature, developed with 2,3-diaminobenzidine tetrahydrochloride (DAB) (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) and counterstained with hematoxylin. The tissue diagnosis and immunostaining results were evaluated by two independent pathologists, and representative images were aquired for data presentation. Written informed consent was obtained from all participants prior to enrollment in the study. The study was approved by the Institutional Human Ethics Committee of the First Hospital of Lanzhou University, Lanzhou, China.

Treatment of cells with celecoxib. The dose- and time-response of SGC7901 and BGC803 cells to celecoxib was examined using various concentrations of celecoxib for various periods of time, as detailed in the relevant figures and figure legends. The effect of celecoxib on the expression of COX-2, NF-κB, Snail and E-cadherin was examined by qPCR and western blot analysis as described below.

qPCR. Total cellular RNA was extracted using a commercial RNA extraction kit. Approximately 30 ng of total RNA was reverse-transcribed into cDNA. The PCR reaction system contained the following: 12.5 μl SYBR® Premix Ex Taq™ II, 0.5 μl of each primer, 2 μl DNA template and 9.5 μl dH_2O. The qPCR conditions were as follows: 95˚C, 5 sec; 62˚C, 30 sec, 40 cycles. The qPCR primers are presented in Table I. Data were analyzed according to the comparative Ct method and were normalized to β-actin expression in each sample.

Western blot analysis. Total cellular protein was extracted using a commercial kit, and the protein concentration was measured using the BCA protein assay (Beyotime Institute of Biotechnology, Haimen, China). Approximately 25 μg of denaturalized protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature before being incubated overnight with primary antibodies against COX-2 (1:500), E-cadherin (1:500), NF-κB (1:500), Snail (1:1000) and β-actin (1:500) at 4˚C. After being washed in TBST three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) (1:5,000) for 1 h at room temperature. The protein bands were then detected using the ECL detection system.

Immunofluorescence and confocal laser scanning microscopy. Cells were harvested at the logarithmic growth phase and placed on glass coverslips in a 6-well plate ($1x10^5$ cells/well) and incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). After treatment with 75 μM celecoxib for 12, 24 and 48 h, the coverslips were washed three times with cold PBS and fixed with cold-acetone:methanol (1:1) for 10 min. The cells were then blocked in 10 g/l BSA solution for 30 min and then incubated with rabbit anti-E-cadherin polyclonal antibody (1:100) at 4˚C overnight. The

Table I. Primer sequences for quantitative PCR.

Primer	Forward	Reverse	Product size (bp)
COX-2	5'-TGGTGCCTGGTCTGATGATGTATGC-3'	5'-ATCTGCCTGCTCTGGTCAATGGAAG-3'	493
E-cadherin	5'-TTAAACTCCTGGCCTCAAGCAATC-3'	5'-TCCTATCTTGGGCAAAGCAACTG-3'	139
NF-κB/p65	5'-TCAGTCAGCGCATCCAGACC-3'	5'-CAGAGCCGCACAGCATTCA-3'	91
Snail	5'-CGCGCTCTTTCCTCGTCAG-3'	5'-TCCCAGATGAGCATTGGCAG-3'	181
β-actin	5'-TGGCACCCAGCACAATGAA-3'	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	186

COX-2, cyclooxygenase-2; NF-κB, nuclear factor-κB.

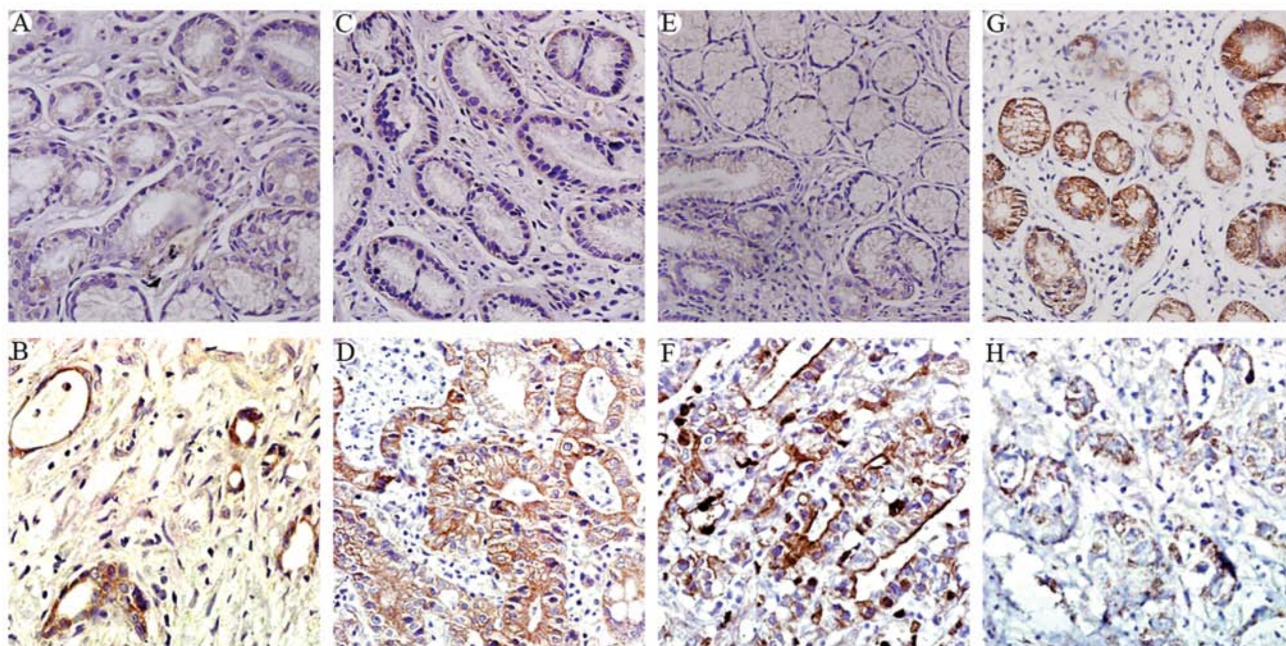

Figure 1. Immunohistochemical staining of (A and B) cyclooxygenase-2, (C and D) nuclear factor-κB p65, (E and F) Snail and (G and H) E-cadherin in normal gastric mucosa (A, C, E and G) and gastric cancer tissues (B, D, F and H). Representative images were shown. Magnification, x100.

cells were then washed three times in PBS and incubated with FITC conjugated goat anti-rabbit antibody (1:200) for 2 h at 37˚C. After three washes in PBS, the coverslips were sealed by glycerol carbonic acid. Fluorescence was observed using a Leica TCS SP2 confocal microscope. PBS was used as the negative control.

Invasion assay. Single cell suspension ($5x10^5$/ml) was prepared with RPMI-1640 supplemented with 1% FBS. Two hundred microliters of cell suspension containing celecoxib (75 μM) were added to the upper chamber of each well. The lower chambers were filled with RPMI-1640 containing 10% FBS. The plates were incubated in a 5% CO_2 container at 37˚C. After 24 h, the cells on the upper membrane surface were removed by wiping with a cotton swab, and the filters were stained with Swiss dye solution for 20 min. The invasive cells on the undersurface of filter were observed under an inverted microscope (x200) and the average number of invasive cells was calculated from five different fields. All assays were performed in triplicate.

Statistical analysis. Data analysis was performed using SPSS11.0 software (IBM SPSS Statistics, Armonk, NY, USA). All data are expressed as the means ± standard deviation. A comparison of the differences between each group was performed using the Student's t-test.

Results

Analysis of the expression of COX-2, NF-κB, Snail and E-cadherin by immunohistochemistry. The gastric cancer tissues generally expressed increased levels of COX-2 (Fig. 1B), NF-κB (Fig. 1D), and Snail (Fig. 1F), compared to the controls (Fig. 1A, C and E, respectively). These proteins were expressed mostly in the cytoplasmic compartments of the tumor cells. By contrast, a high expression of E-cadherin was detected in

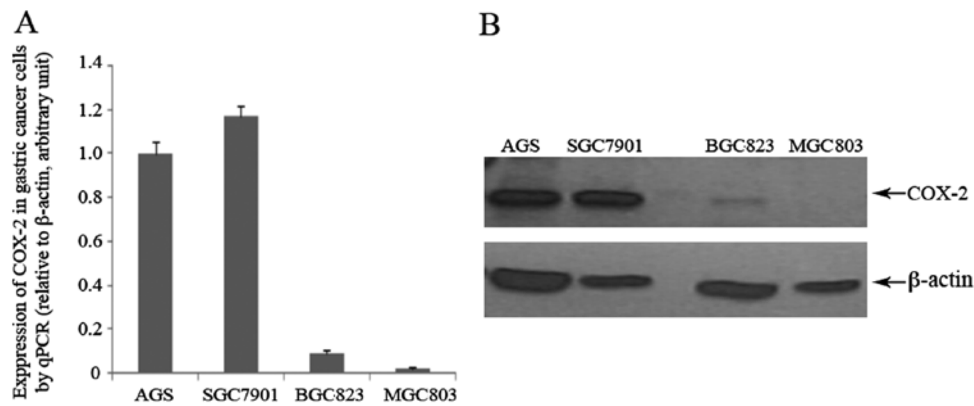

Figure 2. (A) mRNA and (B) protein expression of cyclooxygenase-2 (COX-2) in gastric cancer cells with varying degrees of differentiation.

the normal gastric epithelial tissues, mostly as a membranous protein (Fig. 1G), whereas the expression of E-cadherin was mostly lost in the gastric cancer tissues (Fig. 1H).

Expression of COX-2 in the four gastric cancer cells with varying degrees of differentiation. Using western blot analysis and qPCR, we investigated the expression of COX-2 in the gastric cancer cells with varying degrees of differentiation: AGS (well differentiated), SGC7901 (moderately differentiated), BGC823 (poorly differentiated) and MGC803 cells (undifferentiated). Using qPCR, we observed a gradual decrease in the mRNA expression of COX-2 as the degree of cell differentiation decreased, with the highest expression being found in the SGC7901 cells, and the lowest in the MGC803 cells (Fig. 2A). At the protein level, high levels of COX-2 were observed in the SGC7901 and AGS cells; the BGC823 cells had a very low expression, whereas no COX-2 expression was detected in the MGC803 cells (Fig. 2B). Based on these results, we selected the SGC7901 cells as the COX-2-rich cells and the BGC803 cells as the COX-2-deficient cells for subsequent experiments.

Effect of COX-2 inhibition by celecoxib on the expression of NF-κB, Snail and E-cadherin in the SGC7901 and BGC803 cells. In our previous studies, we demonstrated that COX-2 and NF-κB regulate the expression of E-cadherin via the Snail signaling pathway (10), and that celecoxib restored the lost E-cadherin expression in gastric cancer cells (7). In this study, we treated the SGC7901 and BGC803 cells with celecoxib, and examined its effects on the expression of COX-2, NF-κB, Snail and E-cadherin in gastric cancer cells. Our results may provide clues on the innate correlation between these genes.

When the SGC7901 cells were treated with celecoxib, there was a dose-dependent decrease in the mRNA expression of COX-2, NF-κB and Snail ($P<0.05$, compared to the controls) (Fig. 3A). Further experiments revealed that the treatment of SGC7901 cells with 75 μM of celecoxib for 12, 24 and 48 h led to a marked reduction in the mRNA expression of COX-2, NF-κB and Snail; this reduction occurred in a time-dependent manner, although not for Snail (Fig. 3B) ($P<0.05$, compared to the controls). Notably, treatment of the SGC7901 cells with celecoxib rendered a significant dose- and time-dependent increase in the expression of E-cadherin (Fig. 3A and B) ($P<0.05$, compared to the controls). The increased expression of E-cadherin following treatment with celecoxib was also observed under a confocal fluorescent microscope (Fig. 3C).

By contrast, the same treatment regimen did not alter the mRNA expression of COX-2, NF-κB, Snail and E-cadherin in the BGC803 cells ($P>0.05$, compared to the controls) (Fig. 3D and E).

The above changes were further confirmed at the protein level by western blot analysis. Treatment of the SGC7901 cells with celecoxib caused a dose- and time-dependent decrease (Fig. 3F and G) in the expression of COX-2, NF-κB and Snail. On the other hand, the expression of E-cadherin was increased by celecoxib in a dose- and time-dependent manner (Fig. 3F and G). However, the protein expression of COX-2, NF-κB, Snail and E-cadherin was not significantly altered in the BGC803 cells after the same treatment regimen (Fig. 3H and I) ($P>0.05$, compared to the controls).

Effect of COX-2 inhibition by celecoxib on cell invasion. Treatment of the SGC7901 cells with celecoxib led to an increase in the expression of E-cadherin in the SGC7901 cells ($P<0.05$) (Fig. 3). We then examined the effects of celecoxib on cell invasion. Celecoxib decreased the invasive ability of the SGC7901 cells ($P<0.01$) (Fig. 4).

Effect of COX-2 inhibition by celecoxib on the expression of Akt in SGC7901 cells. Treatment of the SGC7901 cells with celecoxib led to a dose- and time-dependent decrease in the mRNA expression of COX-2 and NF-κB, but not in the expression of Akt (Fig. 5A and B) ($P>0.05$). However, at the protein level, celecoxib caused a dose- and time-dependent decrease in the expression of p-Akt (Fig. 5C and D) ($P<0.05$, compared to the controls), and these changes were in parallel with the altered expression patterns of COX-2 and NF-κB.

Discussion

COX-2 plays an important role in carcinogenesis and metastasis in many types of cancer, including malignancies derived from the gastrointestinal tract (11,12). COX-2 has been reported to induce cell proliferation, inhibit apoptosis and facilitate angiogenesis (5,6). The increased COX-2 expression has been widely reported in gastric cancer; hence, the inhibition of COX-2 has been suggested as a promising approach for the prevention and

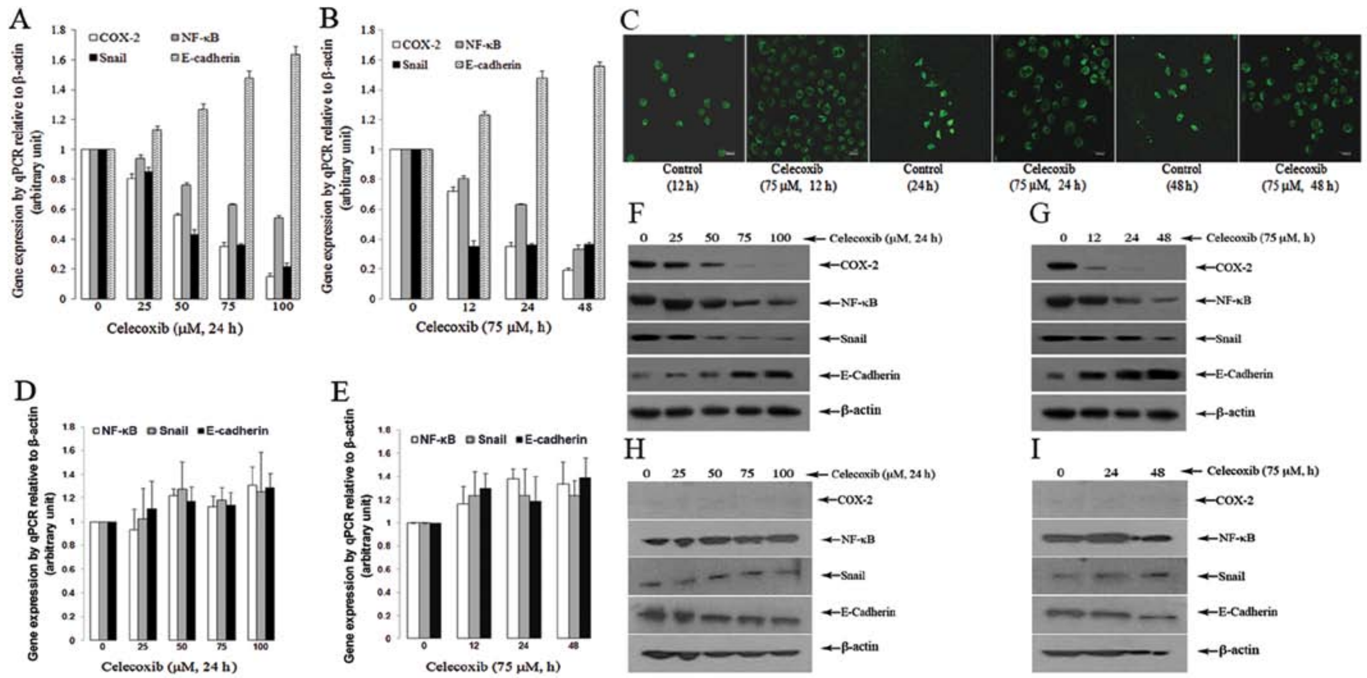

Figure 3. Expression of cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB), Snail and E-cadherin by quantitative PCR (qPCR) in SGC7901 cells treated with (A) various concentrations of celecoxib, or (B) 75 μM of celecoxib for various periods of time. (C) Immunofluorescent staining under a confocal microscope for E-cadherin following celecoxib treatment (magnification, x400). (D and E) The expression of NF-κB, Snail and E-cadherin was also examined by qPCR in the MGC803 cells following the same treatment. The effect of celecoxib on the expression of COX-2, NF-κB, Snail and E-cadherin was further confirmed at the protein level by western blot analysis in (F and G) the SGC7901 cells and (H and I) the MGC803 cells.

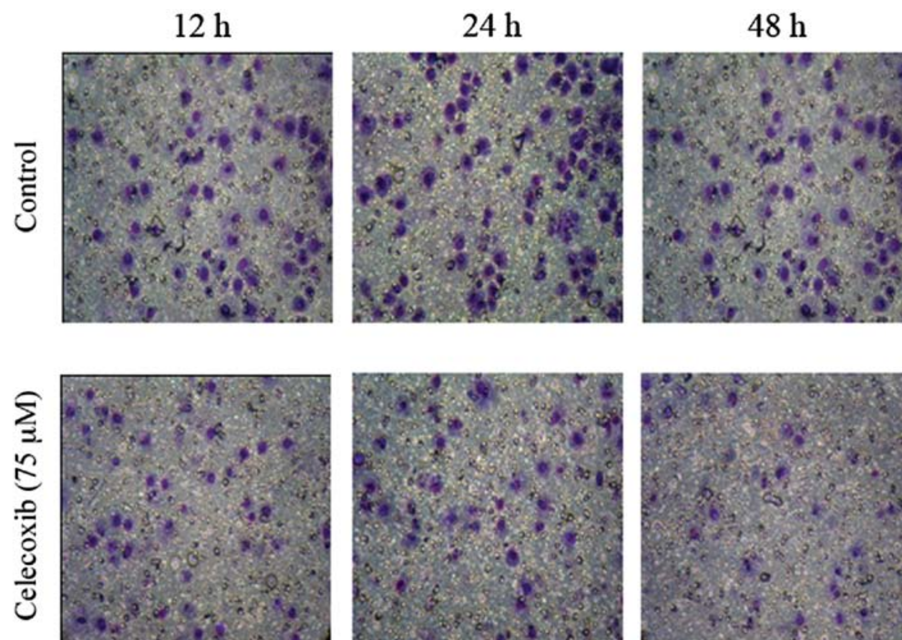

Figure 4. Effect of celecoxib on the invasive ability of the SGC7901 cells. Magnification, x200.

treatment of gastric cancer (13-15). However, the mechanisms through which COX-2 regulates gastric carcinogenesis have not yet been fully elucidated.

The high expression level of COX-2 has been shown to correlate with the downregulation of E-cadherin in prostate cancer (8). E-cadherin is an important molecule in the control of normal cell adhesion and tissue integrity. The loss of E-cadherin has been well recognized in gastric cancer and this has been linked to cancer progression, invasion and metastasis (7,2,16). The pre-operative administration of celecoxib in patients with gastric cancer for seven days has been shown to significantly decrease the expression of COX-2, VEGF and angiogenesis, but increase E-cadherin expression and apoptosis in post-operative gastric cancer tissues (7). In the current

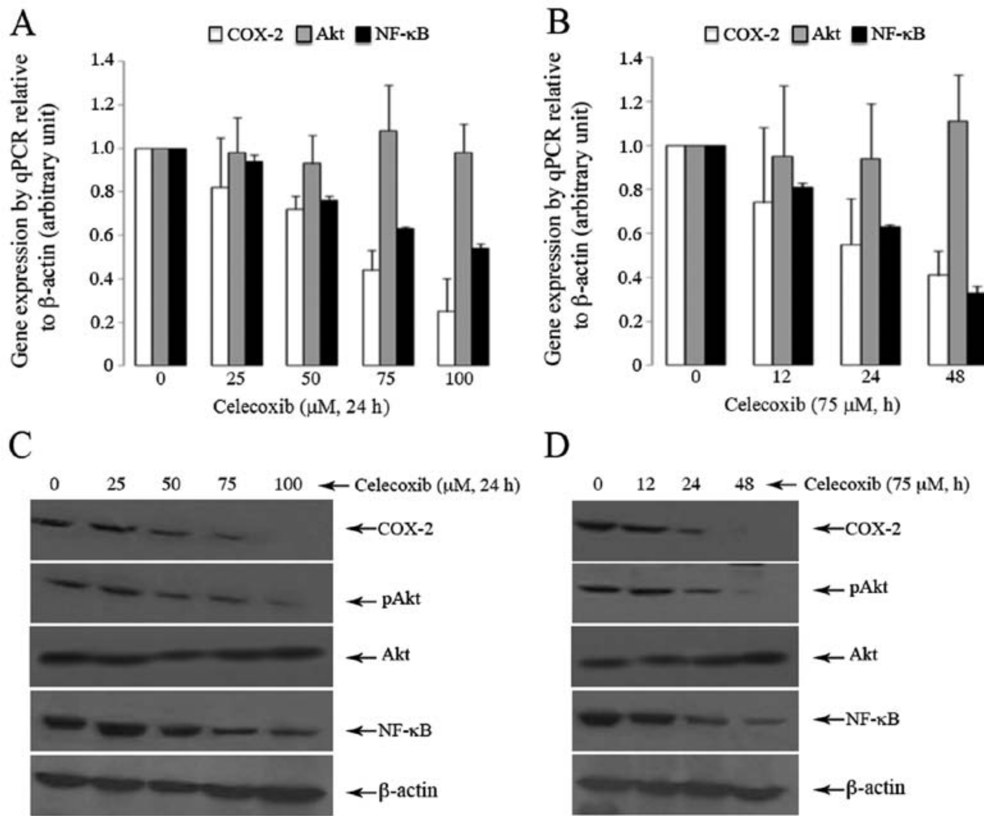

Figure 5. Correlation between the celecoxib-induced alteration in cyclooxygenase-2 (COX-2) and nuclear factor-κB (NF-κB) expression and Akt at (A and B) the mRNA level and (C and D) the protein level in SGC7901 cells.

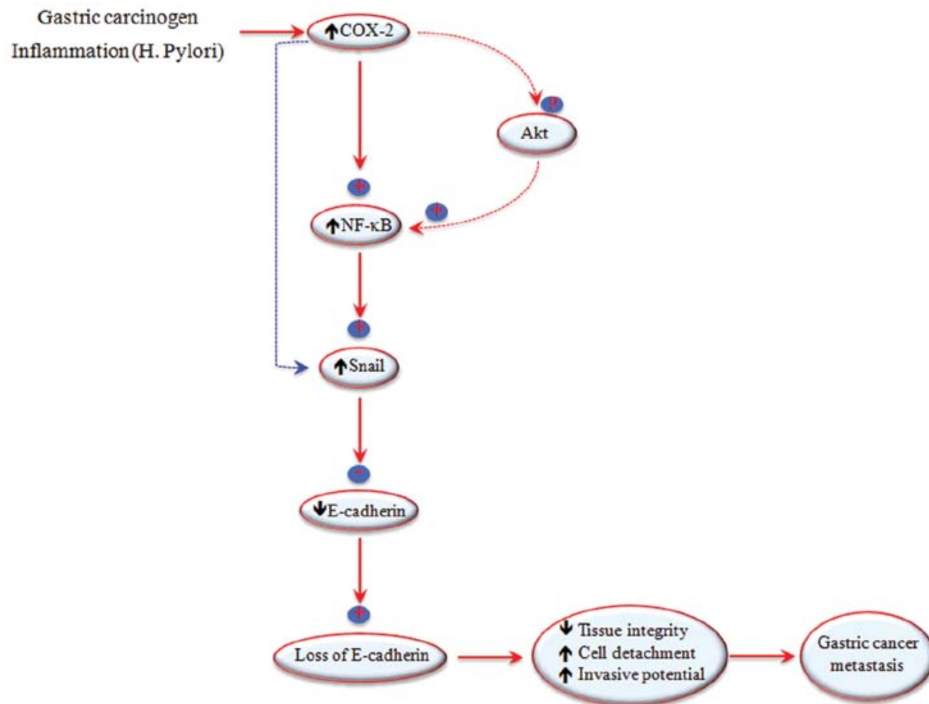

Figure 6. Diagram illustrating the possible molecular mechanisms through which cyclooxygenase-2 (COX-2) interacts with other molecules during the development of gastric cancer metastasis.

study, we observed that celecoxib inhibited COX-2 expression in the SGC7901 cells and that this was associated with the restoration of E-cadherin expression; this in turn, paralleled with a decrease in cell invasion. These data further verify that the role of COX-2 in gastric cancer development is likely associated with the observed loss of E-cadherin expression.

In order to investigate the mechanisms through which COX-2 regulates E-cadherin expression, we measured the expression of Snail, a zinc finger transcription factor of the Snail super family which includes Snail, E12/E47, zinc finger E-box binding homeobox 1 (ZEB1), Smad interacting protein 1 (SIP1) and Slug. Snail binds to the promoters of various effector genes and thereby regulates the transcription and expression of that partner protein (17-19). It has been shown that Snail binds to the E-box of the E-cadherin promoter region and inhibits the transcription and expression of the latter, and thus it is considered a direct inhibitor of E-cadherin (20,21). The downregulation of E-cadherin has indeed been causally linked to the abnormal activity of Snail in several types of cancer (22,23).

In the current study, we observed that COX-2 not only regulates E-cadherin, but also regulates the expression of Snail in gastric cancer cells. Celecoxib, a selective COX-2 inhibitor, was used to treat the SGC7901 cells, in which COX-2 is highly expressed. We found that celecoxib rendered a dose- and time-dependent decrease in the mRNA and protein expression of Snail. By contrast, the BGC803 cells (in which COX-2 is barely detectable) did not show the same response to celecoxib, and this agent did not cause any changes in Snail expression. These data suggest that COX-2 closely correlates with Snail. This is consistent with a previous report on non-small cell lung cancer, showing that COX-2 regulates the expression of E-cadherin via Snail (24). We therefore hypothesized that Snail may be a critical mediator in the COX-2-induced loss of E-cadherin expression in gastric cancer.

Our results revealed that celecoxib inhibited the activity of COX-2 and that this effect was not only associated with the reduced expression of Snail, but also with a marked reduction in the expression of NF-κB subunit p65. Again, in the SGC7901 cells, the mRNA and the protein expression of p65 showed a dose- and time-dependent decrease in response to celecoxib. By contrast, the BGC803 cells did not exhibit a similar response to celecoxib, suggesting that COX-2 regulates the expression of NF-κB.

As NF-κB/p65 directly binds to the promoter region of Snail and thus induces its transcription in cancer (25,26), we hypothesized that in gastric cancer, the interaction between NF-κB/p65 and Snail may play a role in the COX-2-mediated loss of E-cadherin expression. The interaction between COX-2 and NF-κB has not yet been fully elucidated. Previous studies have provided some clues: COX-2 activates IκB kinase (IKK) through the activation of the Akt pathway (27) and the treatment of liver cancer cells with celecoxib has been shown to decrease the phosphorylation level of Akt (28). In this study, we showed that celecoxib did not change the total level of Akt as revealed by qPCR, but induced a time- and dose-dependent downregulation of phosphorylated Akt, and this change was associated with the parallel inhibition of COX-2 and NF-κB. These data suggest that under physiological conditions, COX-2 interacts with its target proteins through the activation of the Akt pathway.

In view of these data, and considering our finding that in gastric cancer tissues, COX-2, NF-κB and Snail showed a very similar expression pattern, and as the same pattern was maintained when the cells were exposed to the COX-2 inhibitor, celecoxib, it can be hypothesized that a complex interaction between COX-2, NF-κB, Snail and E-cadherin exists. These factors may not operate alone during the development of gastric cancer.

In conclusion, based on the published data and those from our study, we suggest that COX-2 activates NF-κB, thus regulating the transcription and expression of E-cadherin through the Snail signaling pathway (Fig. 6), although the direct effect of COX-2 on Snail activation may also play a role in the loss of E-cadherin expression during gastric cancer. Further studies (e.g., experiments involving Snail modulation) are warranted to clarify the mechanisms through which COX-2 interacts with NF-κB, Snail and E-cadherin during the development of gastric cancer.

Acknowledgements

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