PPARγ mediates NSAIDs-induced upregulation of TFF2 expression in gastric epithelial cells

Tadahito Shimada*, Ayako Koitabashi, Yoichiro Fujii, Takashi Hashimoto, Kumi Hosaka, Kyoko Tabei, Takashi Namatame, Masashi Yoneda, Hideyuki Hiraishi, Akira Terano

Department of Gastroenterology and Institute for Medical Science, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

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Abstract Trefoil factor family (TFF) is a group of peptides that play critical roles in maintaining gastric mucosal integrity. In real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) and reporter gene assays, we show that indomethacin and aspirin upregulate TFF2 expression in MKN45 gastric cells. These drugs also activated peroxisome proliferator-activated receptor γ (PPARγ) at concentration ranges that increase TFF2 expression, and upregulated TFF2 expression was suppressed by GW9662, a specific inhibitor of PPARγ. These results suggest that indomethacin and aspirin upregulate gastric expression of TFF2 through activation of PPARγ. This mechanism may be important in reducing the extent of gastric mucosal injury caused by the administration of non-steroidal anti-inflammatory drugs (NSAIDs).

Key words: Trefoil factor family; Cyclooxygenase; Indomethacin; Peroxisome proliferator-activated receptor γ; Gastric epithelial cell

1. Introduction

A number of factors are involved in the defense of gastric mucosa against acid and other endogenous as well as exogenous noxious agents [1]. Among these gastroprotective factors, much attention has been focused on prostaglandins (PGs) and their 'cytoprotective' actions [2–4]. Since non-steroidal anti-inflammatory drugs (NSAIDs), for example aspirin and indomethacin, are inhibitors of cyclooxygenase (COX), a key enzyme for the production of bioactive PGs, administration of NSAIDs is often associated with the induction of gastric mucosal lesions such as erosions and ulcers [5,6]. However, NSAIDs are not always bad for the gastrointestinal tract and recent evidence also shows anti-cancer effects of NSAIDs especially in the colon, suggesting that NSAIDs affect gastrointestinal mucosa in many ways [7,8]. It is also interesting that some of the pharmacological actions of NSAIDs appear to be mediated by COX-independent pathways [9].

Trefoil factor family (TFF) is considered another important gastroprotective factor [10–12]. TFF is a group of protease-resistant polypeptides characterized by a conserved three-loop motif, designated the TFF domain [10,12,13]. Among three known TFF subtypes, TFF1 (pS2) and TFF2 (spasmolytic polypeptide; SP) are expressed in gastric epithelial cells, while TFF3 (intestinal trefoil factor; ITF) expression is found in the goblet cells of the lower intestine [10,11,13]. Although the mode of TFF action is not fully understood, TFF peptides stabilize surface mucus gels by interacting with mucin core proteins and facilitate restitution by promoting cell migration at the site of mucosal injury [10–13]. Konturek et al. recently reported in a rat model that gastric TFF2 expression is upregulated during repeated administration of aspirin [14]. Azarschab et al. also showed that aspirin upregulates TFF2 expression in human gastric cell lines [15]. These findings suggest a possible interaction or cooperation between the PG-COX pathway and TFF peptides in terms of gastric mucosal protection. Since further investigation of this issue may give better understanding of the molecular mechanisms of gastric mucosal protection, we examined the effect of indomethacin and aspirin on TFF expression in gastric epithelial cells. We report here that indomethacin and aspirin upregulate gastric expression of TFF2 through activation of peroxisome proliferator-activated receptor γ (PPARγ).

2. Materials and methods

2.1. Reagents and cell culture

Indomethacin and aspirin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prostaglandin E_2 (PGE_2), arachidonic acid (AA) and GW9662 were purchased from Cayman Chemical (Ann Arbor, MI, USA). To make stock solutions, indomethacin, aspirin, and GW9662 were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich). PGE_2 and AA were dissolved in ethanol. In each experiment, final concentrations of the solvent were less than 0.1% and the solvent itself did not affect the result of the experiment. MKN45 and AGS, cell lines derived from human gastric carcinoma, were obtained from Human Health Resources Bank (Osaka, Japan) and were grown in Ham's F-12 culture medium (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen).

2.2. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Cells grown in 12-well plates were treated with the indicated agents and total RNA was extracted from the cells with Trizol reagent (Invitrogen). First strand cDNA was made with You-Prime first strand beads (Amersham Biosciences; Piscataway, NY, USA) using oligo (dT) primers (Invitrogen). PCR primers used are summarized in Table 1. Real-time quantitative RT-PCR was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems; Foster City, CA, USA) using SYBR green reagents (Applied Biosystems) as described previously [16]. After an initial 10 min at 95°C, the reaction was run for 35 PCR cycles of 15 s at 95°C and 1 min at 56°C. To prepare standard samples, conventional RT-PCR was performed using the primers mentioned above and PCR products were purified with Qiaquick PCR purification kit (Qiagen; Hilden, Germany). Purified PCR products were diluted and used as standard samples (6×10^2 to 6×10^7 copies) for generating a standard curve for each experiment.

*Corresponding author. Fax: (81)-282-86 7761.
E-mail address: tshimada@dokkyomed.ac.jp (T. Shimada).

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Table 1
Primers used for real-time quantitative RT-PCR

TFF1 (GenBank acc. no. NM_003225) (product length 188 bp)
Sense 5′-CAATGGCCACCATGGAGAAC-3′
Antisense 5′-AACGGTGTCGTCGAAACAGC-3′
TFF2 (NM_005423) (product length 161 bp)
Sense 5′-CCAAAGCAAGAGTCGGATCAG-3′
Antisense 5′-CAGTCTTCCACAGACTTCGGG-3′
TFF3 (NM_003226) (product length 158 bp)
Sense 5′-TGTCTGCAAACCAGTGTGCC-3′
Antisense 5′-GCATTCTGTCTTCCTAGTCAGGG-3′
β-actin (NM_001101) (product length 204 bp)
Sense 5′-TTCCTGGGCATGGAGTCCT-3′
Antisense 5′-AGGAGGAGCAATGATCTTGATC-3′

2.3. Reporter vectors and transient transfection assay

To make a TFF2 reporter gene construct, the 5′-flanking region of the human TFF2 gene (GenBank accession number AB038162) was PCR-amplified (−912 to +24) and cloned into the *Sma*I site of the pGL3 basic luciferase reporter vector (Promega; Madison, WI, USA) (TFF2-Luc). A reporter vector for monitoring PPAR activity was made by inserting three times peroxisome proliferator responsive element (PPRE) sequence [17] into the *Sma*I site of the pGL3 basic luciferase vector (PPRE-Luc). Nucleotide identity and direction of the inserts were verified by sequencing of both strands. TFF2-Luc plasmid (0.6 μg/well) or PPRE-Luc plasmid (0.6 μg/well) was transfected into MKN45 cells grown in a subconfluent state in 24-well plates using lipofectamine 2000 reagent (Invitrogen). pSV-β galactosidase control vector (Promega) (0.1 μg/well) was also transfected for standardization. 4 h after transfection, the medium was replaced and test agents were added. 24 h after the addition of the test agents, cells were harvested by lysis buffer, and reporter gene assay was performed in a microplate luminometer LB96V (Berthold Technologies; Bad Wildbad, Germany). Luciferase assay system (Promega) and β-gal reporter gene assay kit (Roche Diagnostics; Mannheim, Germany) were used to measure firefly luciferase activity and galactosidase activity, respectively.

2.4. Statistics

Data are presented as the mean ± S.D. Statistical differences between two groups were analyzed by unpaired *t*-test. Analysis of variance (ANOVA) was performed when more than two groups were compared, and when significant ($P < 0.05$), Scheffes multiple comparison test was applied to test for the differences between individual groups. A P value < 0.05 was considered to be significant.

3. Results

3.1. Effect of indomethacin on the expression of endogenous TFF mRNA in MKN45 cells

Although MKN45 cells mainly expressed TFF1 and TFF2, TFF3 mRNA was also detected by real-time quantitative RT-PCR experiments as described in the previous paper [18]. To examine the effect of indomethacin on endogenous TFF mRNA expression, MKN45 cells were treated with different concentrations of indomethacin (0–250 μM) for 24 h and the expression of each TFF subtype was analyzed by real-time quantitative RT-PCR. As shown in Fig. 1, indomethacin (30–250 μM) significantly upregulated TFF2 mRNA expression in a dose-dependent manner (Fig. 1B). High concentrations of indomethacin also slightly upregulated TFF1 mRNA expression (Fig. 1A). However, TFF3 mRNA expression was not significantly affected (Fig. 1C). In the following experiments, we focused on the effect of indomethacin on TFF2 expression. Fig. 1D shows a time-course experiment showing the effect of indomethacin (125 μM) on the expression of TFF2 mRNA. Statistically significant upregulation was observed after a 6-h treatment with indomethacin.

3.2. Effect of indomethacin on the transcription of the TFF2 reporter gene

Transient transfection assay was performed to confirm the effect of indomethacin on TFF2 transcription. After TFF2-Luc was transfected to MKN45, cells were treated with indomethacin (0–250 μM) for 24 h. As shown in Fig. 2A, TFF2 transcription was upregulated by treatment with indomethacin in a dose-dependent manner. Similar to the PCR experiment described above, significant upregulation was observed at 30–250 μM of indomethacin. Since indomethacin is a potent in-

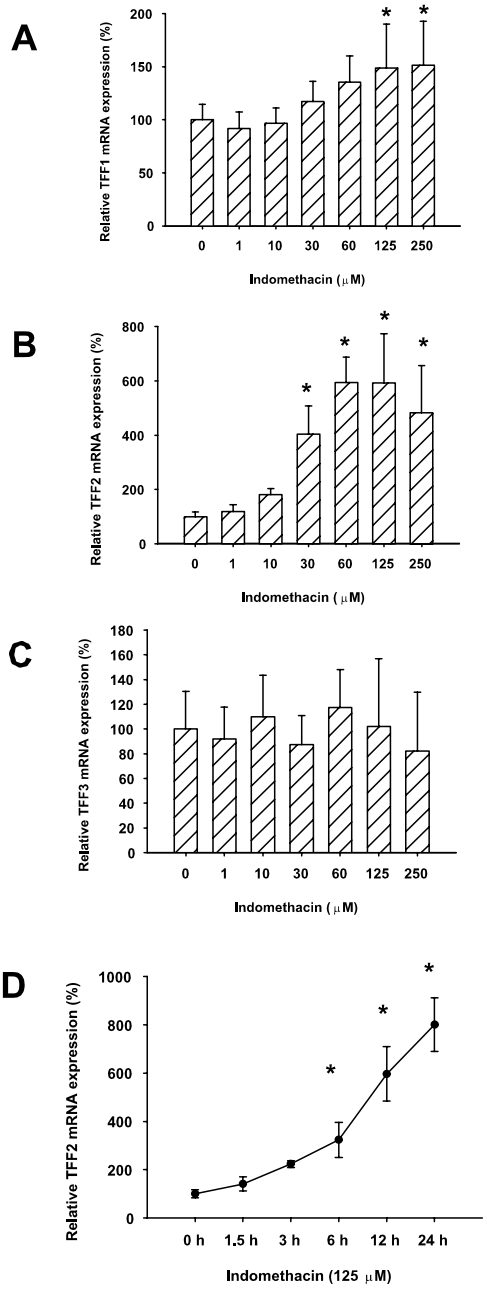

Fig. 1. A–C: Quantitative RT-PCR experiments showing the effects of indomethacin (0–250 μM, 24-h incubation) on the expression of endogenous TFF1 (A), TFF2 (B), and TFF3 (C) mRNA in MKN45 gastric cells ($n = 4$). *$P < 0.01$ vs. control. D: A representative time-course experiment showing the effect of indomethacin (125 μM) on the expression of endogenous TFF2 mRNA in MKN45 cells ($n = 4$). *$P < 0.01$ vs. control.

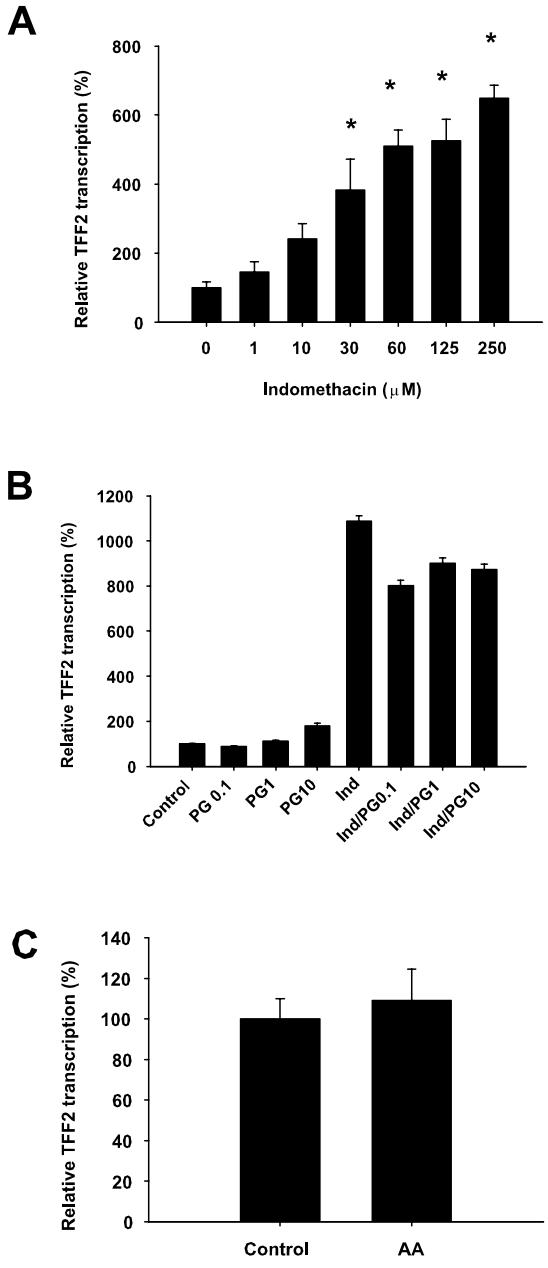

Fig. 2. A: Effect of indomethacin (0–250 μM, 24-h incubation) on the transcription of TFF2 reporter gene ($n=4$). *$P<0.01$ vs. control. B: Effect of externally applied PGE_2 (0.1–10 μM) on indomethacin (Ind) (125 μM, 24-h incubation)-induced upregulation of TFF2 reporter gene transcription ($n=4$). C: Effect of externally applied AA (30 μM, 24-h treatment) on the transcription of the TFF2 reporter gene ($n=4$).

hibitor of COX, we examined the effect of externally applied PGE_2 on the indomethacin-induced upregulation of TFF2 transcription. MKN45 cells were pre-incubated with PGE_2 (0.1–10 μM) for 2 h prior to the addition of indomethacin (125 μM), and indomethacin was treated in the presence of PGE_2 for 24 h. As shown in Fig. 2B, PGE_2 itself had a slight stimulatory effect on TFF2 transcription at 10 μM. Although indomethacin-induced upregulation of TFF2 transcription was partially suppressed by PGE_2, most of the indomethacin effect remained, suggesting that PG depletion by indomethacin is not a main cause of TFF2 upregulation. We also tested the effect of AA, because there is a possibility that AA accumulates when COX activity is suppressed by indomethacin. However, externally applied AA (30 μM, 24-h incubation) had no significant effect on TFF2 transcription (Fig. 2C).

3.3. *Indomethacin-induced PPARγ activation in MKN45 cells and the effect of GW9662 on indomethacin-induced upregulation of TFF2 transcription*

Indomethacin has been reported to act as a ligand for PPARγ [19]. Since we have previously reported the expression

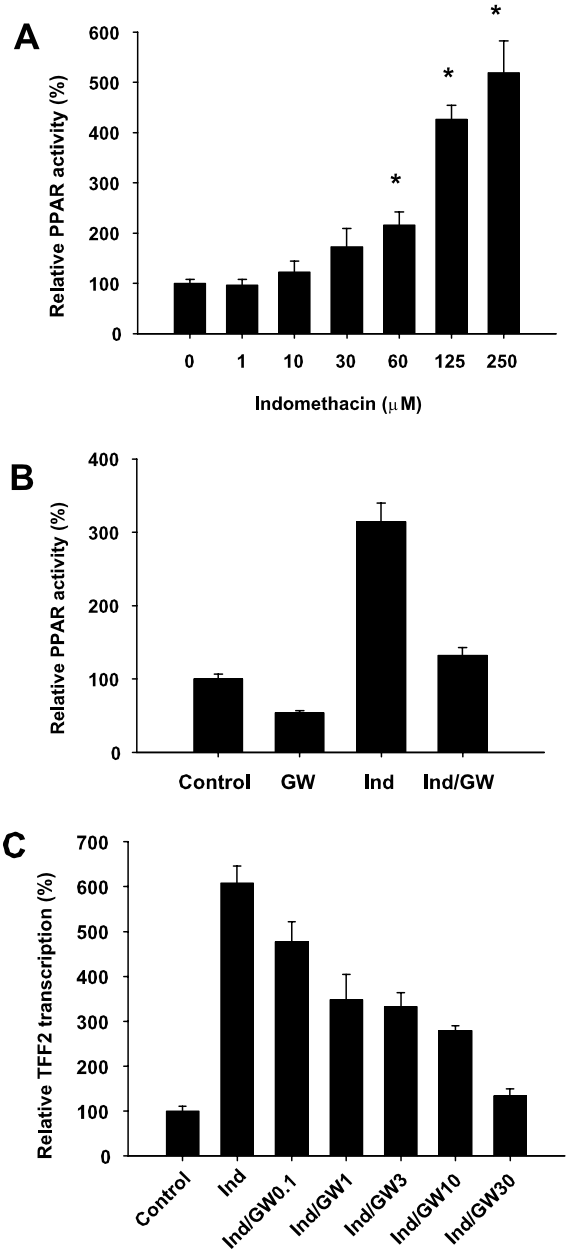

Fig. 3. A: Effect of indomethacin (0–250 μM, 24-h incubation) on the transcription of PPRE-driven reporter gene ($n=4$). *$P<0.01$ vs. control. B: Effect of GW9662 (GW) (30 μM) on indomethacin (Ind) (125 μM, 24-h incubation)-induced upregulation of the transcription of PPRE-driven reporter gene ($n=4$). C: Effect of GW9662 (GW) (0.1–30 μM) on indomethacin (Ind) (125 μM, 24-h incubation)-induced upregulation of the transcription of TFF2 reporter gene ($n=4$).

of PPARγ in MKN45 cells [20], we tested whether indomethacin really activates PPARγ in this cell type. As shown in Fig. 3A, indomethacin (30–250 μM) stimulated the transcription of the PPRE-Luc reporter gene in a dose-dependent manner and it was suppressed by GW9662 (30 μM), a specific inhibitor of PPARγ [21] (Fig. 3B), confirming the activation of PPARγ by indomethacin in MKN45 cells. Thus, we examined the possible involvement of PPARγ in the indomethacin-induced upregulation of TFF2 transcription. As shown in Fig. 3C, indomethacin (125 μM)-induced upregulation of TFF2 transcription was suppressed by GW9662 in a dose-dependent manner, suggesting that indomethacin modulates TFF2 transcription through PPARγ.

3.4. *Effects of aspirin on PPARγ activity and TFF2 transcription in MKN45 cells*

Although aspirin and salicylate are not regarded as ligands for PPARγ [19], Azarschab et al. reported the upregulation of TFF2 expression by aspirin at mM concentration ranges [15]. Thus, we next examined whether high concentrations of aspirin affect PPARγ activity in MKN45 cells. As shown in Fig. 4A, aspirin at 5 mM caused about a 2.7-fold increase in PPAR-Luc transcription and it was sensitive to GW9662 (30 μM), suggesting that PPARγ is activated in response to aspirin treatment. Fig. 4B shows the effect of aspirin (5 mM) and GW9662 (30 μM) on TFF2 transcription. We found that TFF2 transcription was upregulated by aspirin and this upregulation was suppressed by GW9662. These results suggest that, similar to indomethacin, aspirin affects TFF2 expression through PPARγ.

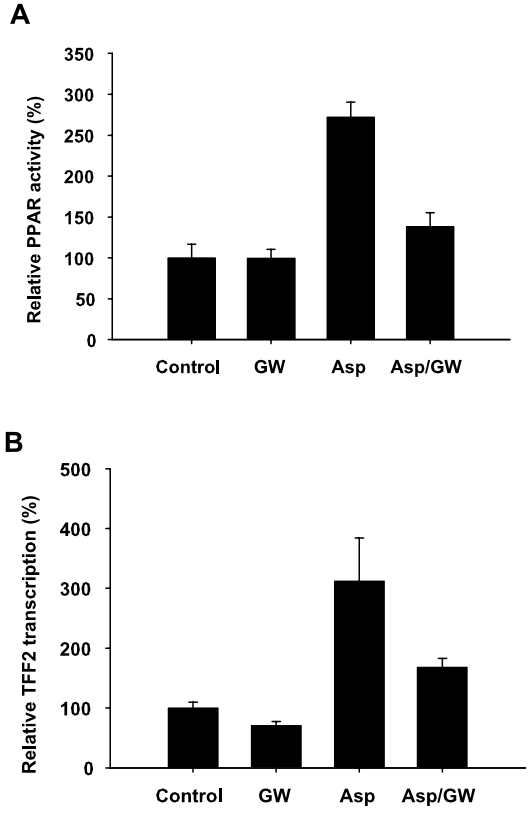

Fig. 4. A: Effects of aspirin (Asp) (5 mM, 24-h incubation) and GW9662 (GW) (30 μM) on the transcription of PPRE-driven reporter gene ($n = 4$). B: Effects of aspirin (Asp) (5 mM, 24-h incubation) and GW9662 (GW) (30 μM) on the transcription of TFF2 reporter gene ($n = 4$).

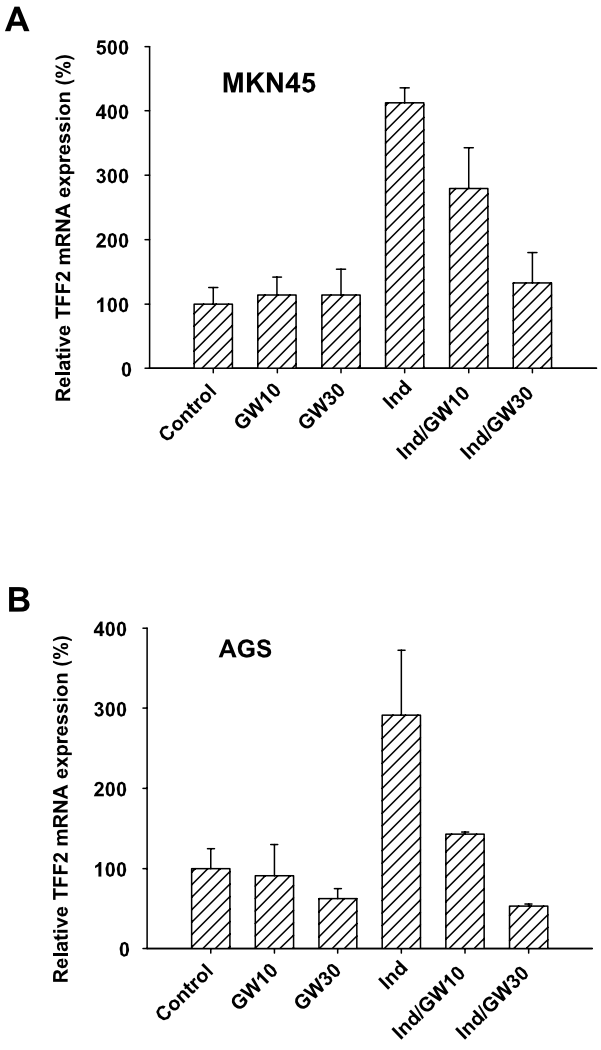

Fig. 5. Quantitative RT-PCR experiments showing the effects of indomethacin (Ind) (125 μM, 24-h incubation) and GW9662 (GW) (10–30 μM) on the expression of endogenous TFF2 mRNA in MKN45 cells (A) ($n = 4$) and AGS cells (B) ($n = 4$).

3.5. *Effect of indomethacin and GW9662 on the expression of endogenous TFF2 mRNA in MKN45 and AGS cells*

Finally, we returned to RT-PCR experiments to determine whether endogenous TFF2 mRNA expression is modulated by indomethacin through a similar mechanism. Fig. 4A shows the effect of GW9662 on indomethacin-induced upregulation of TFF2 mRNA expression in MKN45 cells. Similar to the reporter gene experiments, GW9662 suppressed the indomethacin-induced upregulation of TFF2 mRNA expression in a dose-dependent manner. We also tested another gastric cell line, AGS, and found that GW9662 suppressed indomethacin-induced upregulation of TFF2 mRNA expression (Fig. 5B). Thus, the involvement of PPARγ in the action of indomethacin is not unique to MKN45.

4. Discussion

Genes encoding human TFF are clustered on chromosome

21q22.3 [22] and a number of studies have reported the regulatory mechanisms of TFF gene expression [12,13]. Gott et al. showed the presence of a TATA box and other potential binding sites for Myc, PEA3, and Ets-like factor in the 5′-flanking region of the TFF2 gene [22]. GATA-6 binding sites are also considered to play a basic role in the stomach-specific expression of TFF2 [23]. Azarschab et al. recently examined the effect of aspirin on the expression of TFF in gastric cancer cell lines, MKN45 and Kato3, and found that aspirin upregulates TFF2 expression. Since they observed that staurosporine, an inhibitor of protein kinases including protein kinase C, abolished this effect of aspirin, they speculated that protein kinase C is involved in the aspirin-induced upregulation of TFF2 expression.

In the present study, we analyzed the mechanism of the indomethacin-induced TFF2 expression in MKN45 cells. Since externally applied PGE_2 had little effect on indomethacin-induced TFF2 expression, the depletion of PG by indomethacin was not likely to be a main trigger of TFF2 upregulation. Although it is generally accepted that most of the pharmacological actions of NSAIDs arise from inhibition of COX and suppression of PG synthesis, evidence also suggests the presence of possible targets of NSAIDs other than COX [9]. Aspirin, for example, is known to inhibit the activity of certain transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [9], and aspirin also affects the activity of cellular kinases, such as mitogen-activated protein (MAP) kinases and cyclin-dependent kinases [9]. It has been reported that indomethacin induces the expression of a member of the TGF superfamily, designated NAG-1 (NSAIDs-activated gene-1) [24] and also induces the expression of a member of the nuclear receptor family, nerve growth factor-inducible B (NGFI-B) [25]. More importantly, Lehmann et al. showed that indomethacin and some other NSAIDs can act as ligands for PPARγ [19]. PPAR is a member of the nuclear receptor superfamily and consists of three subtypes, PPARα, PPARδ, and PPARγ [26]. Among these subtypes, PPARγ is expressed at a high level in adipose tissue and is critically involved in the adipocyte differentiation and fatty acid metabolism [26].

Gastrointestinal epithelial cells also express PPARγ at a significant level and a number of studies have suggested the involvement of PPARγ in the regulation of cell proliferation, differentiation, and cell death of gastrointestinal epithelial cells [26]. We have previously reported the expression and functions of PPARγ in colon cancer cell lines and gastric cancer cell lines, including MKN45 [16,20]. Recently, we have observed the induction of TFF2 expression in MKN45 cells by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and troglitazone, which are established ligands for PPARγ [26]. In the present study, we showed that indomethacin activates PPARγ in MKN45 cells, and we found that indomethacin-induced TFF2 expression is sensitive to GW9662, a specific inhibitor of PPARγ. Thus, it is very likely that indomethacin upregulates TFF2 expression through PPARγ. Although aspirin has not been considered a ligand for PPARγ [19,9], we also found in the present study that aspirin activates PPARγ at high concentrations, and that GW9662 suppresses aspirin-induced TFF2 expression. Therefore, although possibility of the involvement of other signaling pathways is not completely excluded, we hypothesize that PPARγ mainly mediates the action of aspirin on TFF2 expression. Azarschab et al. [15] mapped an aspirin responding element to −546 to −758 bp upstream of the TATA box of the human TFF2 gene. Since apparent PPRE is not present in the 5′-flanking region of the human TFF2 gene, the effect of PPARγ may be indirect. We are currently investigating the PPARγ action on the TFF2 gene transcription in more detail.

Babyatsky et al. [27] and McKenzie et al. [28] showed a gastroprotective role of oral or topically administered TFF2 peptides in rats. Farrell et al. recently developed TFF2-deficient mice and found that these mice are more susceptible to indomethacin-induced gastric injury [29]. Konturek et al. reported that repeated administration of aspirin leads to the upregulation of TFF2 expression and resistance to the aspirin-induced gastric mucosal injury in rats [14]. Collectively, the indomethacin- or aspirin-induced upregulation of TFF2 expression shown in the present study is likely to be an important adaptive mechanism to reduce the extent of NSAIDs-induced gastric injuries.

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