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RESEARCH PAPER

Somatostatin inhibits colon cancer cell growth through cyclooxygenase-2 downregulation

R Colucci[1], C Blandizzi[1], N Ghisu[1], T Florio[2] and M Del Tacca[1]

[1]*Department of Internal Medicine, Division of Pharmacology and Chemotherapy, University of Pisa, Pisa, Italy and* [2]*Department of Oncology, Biology and Genetics, Section of Pharmacology, University of Genova, Genova, Italy*

Background and purpose: Cyclooxygenase-2 (COX-2) is expressed in colonic neoplasms, where it supports cell proliferation via prostaglandin E_2 (PGE_2) production. This study investigated the effects of somatostatin-14 on COX-2 expression, PGE_2 production and proliferation in colon cancer cells.
Experimental approach: Human colon adenocarcinoma cell lines Caco-2, HT-29 and HCT116 were used. The following techniques were employed: colourimetric assay for cell growth; 5-bromo-2′-deoxyuridine assay for DNA synthesis; enzyme immunoassay for PGE_2; COX-2 mRNA silencing; RT–PCR or Western blot for somatostatin receptor subtypes, cyclooxygenase isoforms, phosphorylated-ERK-1/ERK-2 and phosphorylated-Akt.
Key results: HT-29 and Caco-2 cells expressed COX-2 and somatostatin receptors ($sst_{3/4/5}$ and $sst_{3/5}$, respectively). HCT116 cells did express somatostatin receptors ($sst_{2/3/5}$), but not COX-2. Somatostatin-14 inhibited basal COX-2 expression, PGE_2 production, DNA synthesis and growth in Caco-2 cells and these effects were prevented by BN81658 (sst_3 receptor antagonist). Basal proliferation of HT-29, HCT116 and COX-2-silenced Caco-2 cells was not affected by somatostatin-14. Stimulation of HT-29 cells with gastrin-17 elicited increments of ERK-1/ERK-2 and Akt phosphorylation, COX-2 expression, PGE_2 production, DNA synthesis and cell growth, which were all counteracted by somatostatin-14. Somatostatin-14-induced inhibition of COX-2 expression, PGE_2 production and DNA synthesis were blocked by BIM23056 (sst_5 receptor antagonist).
Conclusions and implications: Somatostatin decreases COX-2 expression and function in colon cancer cells via activation of sst_3 or sst_5 receptors, and these effects contribute to the inhibitory action of somatostatin on cell proliferation. These findings can be relevant to the development of therapeutic strategies based on the modulation of the COX-2 pathway.
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Keywords: somatostatin; somatostatin receptor; cyclooxygenase-2; prostaglandin E_2; gastrin; colon cancer

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; EGF, epidermal growth factor; ERK-1/ERK-2, extracellular-regulated kinases; FBS, foetal bovine serum; MAPK, mitogen-activated protein kinase; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol-3 kinase; RT–PCR, reverse transcription–PCR

Introduction

Colorectal cancer represents one of the most common malignancies worldwide, and despite advances in chemotherapy, this neoplasia remains a major cause of death. High risks of severe adverse effects are also associated with available anti-cancer drugs (Kelly and Goldberg, 2005). For these reasons, strong efforts are currently being made to search for novel pharmacological therapies and, among different options, the roles played by cyclooxygenase (COX) pathways and gut hormones in the control of colorectal cancer growth represent areas of active investigation (Brown and DuBois, 2005). Studies on individuals under treatment with non-steroidal anti-inflammatory drugs have shown a reduction in colorectal cancer mortality compared to untreated subjects, and there is evidence that the reduced risk depends on the pharmacological modulation of COX, the dominant enzyme on the metabolic pathway that converts arachidonic acid into prostanoid mediators (Thun *et al.*, 1991; Chan *et al.*, 2007). In particular, after cloning and molecular characterization of two COX isoforms (COX-1, COX-2), COX-2 was found in colonic neoplasms, where it actively contributed to support cell migration, proliferation and neovascularization (Eberhart *et al.*, 1994). On this basis, pharmacological strategies designed to downregulate COX-2 expression and/or enzyme function in colorectal cancer cells are expected to exert beneficial effects (Chun and Surh, 2004).

Somatostatin belongs to a family of regulatory peptides expressed in the central nervous system and the digestive

Correspondence: Professor C Blandizzi, Divisione di Farmacologia e Chemioterapia, Dipartimento di Medicina Interna, Università di Pisa, Via Roma 55, 56126 Pisa, Italy.
E-mail: c.blandizzi@med.unipi.it
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tract. This peptide is characterized by a wide spectrum of biological actions, including modulation of cell growth, which are mediated by five receptor subtypes, named sst_1 to sst_5 (Weckbecker *et al*., 2003; Alexander *et al*., 2008). Somatostatin can exert tumour inhibitory effects through several different mechanisms: (1) interference with growth factors and hormones acting as stimulants of cell growth; (2) inhibition of tumour neoangiogenesis; (3) direct anti-proliferative effects through sst receptors located on neoplastic cells (Dasgupta, 2004). When considering the gastrointestinal system, a significant expression of sst receptors has been detected in normal and malignant human colorectal tissues (Laws *et al*., 1997; Vuaroqueaux *et al*., 2000). In addition, somatostatin and its analogues inhibited the proliferation of *in vitro* or xenografted human colon cancer cell lines (Dy *et al*., 1992).

Clinical studies suggest that hypergastrinaemia is associated with an increased risk of colorectal cancer, and it has been observed that most patients with colorectal cancer may present an increased expression of gastrin receptors and COX-2 in their neoplastic tissues (Thorburn *et al*., 1998; Hartwich *et al*., 2001). Moreover, gastrin has been shown to stimulate the growth of human colon cancer cells via gastrin receptor-mediated COX-2 induction and increase in prostaglandin E_2 (PGE_2) production (Colucci *et al*., 2005). In the present study, three human colon cancer cell lines, HT-29, Caco-2 and HCT116, were examined for the expression of sst receptor subtypes, and experiments were designed to pursue the following aims: (1) to examine whether somatostatin affects cell proliferation via modulation of COX-2; (2) to characterize the mechanisms underlying the interaction between sst receptors and the COX-2 pathway in cancer cells.

Methods

Cells

The human colon adenocarcinoma cell lines, HT-29, Caco-2 and HCT116 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in McCoy's medium (Invitrogen srl, Milan, Italy), containing 10% foetal bovine serum (FBS) and supplemented with penicillin and streptomycin (both at 2%). Cells were maintained in a humidified incubator at 37 °C, in the presence of 5% CO_2 and were sub-cultured following enzymatic digestion by trypsin/EDTA. For experimental procedures, cells (5×10^4 per well) were seeded in 96-well plates in 10% FBS-containing medium for 24 h. Cells were then washed with phosphate-buffered saline and serum-free medium was added. The day after, test drugs were added to the culture medium for periods of 24 or 72 h. Serum-free medium containing test drugs was replaced every 24 h. In preliminary experiments, the expression of gastrin/CCK_2 receptors was examined in these cell lines. Assays of reverse transcription-polymerase chain reaction (RT–PCR) revealed the presence of mRNA transcripts for gastrin/CCK_2 receptors in HT-29 cells, but not in Caco-2 and HCT116 cells.

Assays for cell growth and DNA synthesis

Cell growth was determined by means of a kit for colourimetric assay, based on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-4-sulphophenyl)-2H-tetrazolium (CellTiter 96 AQ_{ueous} One Solution Proliferation Assay, Promega, Madison, WI, USA), in accordance with manufacturer's instructions. In control experiments, cells were collected by trypsinization and subjected to direct count. DNA synthesis was determined by measuring incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA using a cell proliferation kit (Roche Diagnostics, Mannheim, Germany). For this purpose, BrdU (10 μM) was added to culture medium after treatment with test drugs. After 6 h, cells were fixed and BrdU incorporation was determined by enzyme-linked immunosorbent assay in accordance with manufacturer's instructions. In all cases, experiments were performed in triplicate.

Assay of PGE_2

Levels of PGE_2 released into the culture medium were determined by means of a kit for competitive enzyme-linked immunoassay (Cayman Chemical, Ann Arbor, MI, USA). Experiments with test drugs were designed as reported above for proliferation studies. At the end of incubation, aliquots of culture medium (0.5 mL) were collected into ice-cold vials containing indomethacin 1 mM (final concentration) and stored at −80 °C until the subsequent analysis. Cells were then collected by trypsinization and counted. At the time of immunoenzyme assay, carried out in accordance with manufacturer's instructions, each sample was diluted 1:10 (v/v) and assayed in triplicate. PGE_2 levels were expressed as pg 10^{-5} cells.

COX-2 mRNA silencing

Silencing of COX-2 mRNA expression was performed in accordance with the procedure reported by Charames and Bapat (2006). Cells were transfected with COX-2 silencing oligonucleotide (small-interfering RNA, siRNA) in Lipofectamine 2000 (Invitrogen srl) reagent according to the manufacturer's instructions. Lipofectamine 2000 reagent (20 μL of) were incubated with 500 μL of medium for 5 min. Subsequently, a mixture of 20 μL of siRNA in annealing buffer and 500 μL medium was added. After incubation for 20 min at room temperature, the combined mixture was dropwise added to each well, resulting in a siRNA concentration of 10 nM. The COX-2 siRNA was synthesized (Qiagen, Milan, Italy) according to the cDNA sequence (5′-AAACTGCTCAAC ACCGGAATTTTT-3′, nucleotides 291–313). A non-specific control (non-silencing) siRNA against the target sequence (5′-AATTCTCCGAACGTGTCACGT-3′) was also employed.

RNA extraction and RT–PCR

The expression of mRNA encoding sst receptors was assessed by RT–PCR. Total RNA was isolated from cells by means of Trizol (Invitrogen srl) and chloroform, and treated with DNAase. Following DNAase inactivation, 1 μg of total RNA served as template for single-strand cDNA synthesis in a reaction using 2 μL random hexamer oligonucleotide primers (0.5 μg μL^{-1}) with 200 U of MMLV-RT in manufacturer's buffer containing 500 μM deoxynucleotide triphosphate

mixture (dNTP) and 10 mM dithiothreitol. cDNA samples were subjected to PCR in the presence of specific oligonucleotide primers based on the nucleotide sequence of sst receptors (Eden and Taylor, 1993; Yamada *et al*., 1993). PCR, consisting of 5 μL of RT products, Taq polymerase 2.5 U, dNTP 100 μM and oligonucleotide primers 0.5 μM, was carried out by a PCR-Express thermocycler (Hybaid, Ashford, Middlesex, UK). The following standard conditions were used: 3 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final extension period of 7 min at 72 °C. Aliquots of RNA not subjected to RT were included in PCR reactions to verify the absence of genomic DNA. The efficiency of RNA extraction, RT and PCR was evaluated by specific sets of oligonucleotide primers for the constitutively expressed human β-actin gene. cDNA products (expected size: sst_1, 237 bp; sst_2, 234 bp; sst_3, 228 bp; sst_4, 234 bp; sst_5, 225 bp; β-actin, 286 bp) were separated by 1.8% agarose gel electrophoresis, in a Tris-buffer 40 mM containing 2 mM EDTA, 20 mM acetic acid (pH 8) and stained with ethidium bromide. cDNA bands were then visualized by ultra violet light.

Western blot

Western blot analysis was used to examine the protein expression of sst receptor subtypes and COX isoforms (COX-1, COX-2) as well as the phosphorylation status of regulatory proteins belonging to the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways. Cells were lysed on ice in radioimmune precipitation assay buffer. Lysates were then centrifuged at 15 000 *g* for 10 min at 4 °C. The supernatants were separated from pellets and stored at −80 °C. Proteins extracted from rat brain cortex were used as positive controls in experiments designed to assess the expression of sst receptors (Kumar *et al*., 1999). Protein concentration was determined by Bradford method (Bio-Rad protein assay reagent, Hercules, CA, USA). Equivalent amounts of protein lysates (30 μg) were denatured for 5 min, separated by electrophoresis on SDS–polyacrylamide gel electrophoresis (10%) and transferred onto a nitrocellulose membrane. The blots were blocked overnight with 5% non-fat-dried milk in Tris-buffered saline Tween-20 (TBS-T), and incubated with primary antibodies raised against sst_1, sst_2, sst_3, sst_4, sst_5 receptors, COX-1 or COX-2. Phosphorylation of extracellular-regulated kinases (ERK-1/ERK-2) and Akt were evaluated by incubation of blots with anti-phospho-p42/p44 MAPK (Thr202/Tyr204) and antiphospho-Akt (Ser473) antibodies. After washings with TBS-T, blots were incubated with a peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature. Following washings with TBS-T, the immunoreactive bands were visualized by incubation with chemiluminescent reagents, and exposed to Kodak Image Station 440 for signal detection and densitometric analysis. Blots were then stripped and reprobed with anti-β-actin antibody. The relative intensity of immunoreactive bands was normalized to that of β-actin.

Statistical analysis

Results are given as mean ± s.e.mean. The significance of differences was evaluated by one-way analysis of variance for unpaired data followed by *post hoc* analysis by Dunnett's or Bonferroni's test, as appropriate. *P*-values lower than 0.05 were considered significant; '*n*' indicates the number of experiments.

Drugs and reagents

The following drugs and antibodies were used: human gastrin-17, human somastostatin-14, human epidermal growth factor, SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole), BIM23056, sodium orthovanadate, GF109203X, indomethacin (Sigma Chemicals Co., St Louis, MO, USA); phorbol myristate acetate, KT-5823 (9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b-11a-triazadibenzo(a,g)-cycloocta(cde)-trinden-1-one; EMD Biosciences Inc., San Diego, CA, USA); PGE_2 (Cayman Chemical); L-745337 (5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone; kindly provided by Merck Frosst Canada Inc., Dorval, Canada); L-803087 trifluoroacetate (Tocris Bioscience, Bristol, UK); BIM23206 and BN81658 (kindly provided by MD Culler, Biomeasure Incorporated/IPSEN, Milford, MA, USA); L-796778 (kindly provided by S Rohrer, Merck Research Laboratory, Boston, MA, USA) anti-sst_1, anti-sst_2, anti-sst_3, anti-sst_4, anti-sst_5, anti-COX-1, anti-COX-2 and anti-β-actin antibodies (Santa-Cruz Biotechnology Inc.); anti-phospho-p42/44 MAP-kinase and anti-phospho-Akt antibodies (Cell Signalling Technology, Beverly, MA, USA). Receptor nomenclature follows that recommended by Alexander *et al*. (2008).

Results

Expression of somatostatin receptor subtypes and cyclooxygenase isoforms

Reverse transcription–PCR assays showed the presence of mRNA coding for sst_3, sst_4 and sst_5 receptors in HT-29 cells; sst_3 and sst_5 receptors in Caco-2 cells and sst_2, sst_3 and sst_5 receptors in HCT116 cells (Figure 1). Western blot analysis confirmed the expression of sst_3, sst_4 and sst_5 receptors in HT-29 cells; sst_3 and sst_5 receptors in Caco-2 cells; sst_2, sst_3 and sst_5 receptors in HCT116 cells, and showed the presence of all five sst receptors in rat brain cortex (Figures 2a and b). The patterns of immunoreactive bands were consistent with data reported for the expression of sst receptor subtypes in humans and rats (Kumar *et al*., 1999; Reynaert *et al*., 2001). Of note, the protein expression of sst_5 receptors was predominant in HT-29 and HCT116 cells over Caco-2 cells, whereas there was a predominance of sst_3 receptor expression in Caco-2 cells over HT-29 and HCT116 cells (Figures 2a and b). Western blot showed also that both COX-1 and COX-2 proteins are constitutively expressed in HT-29 and Caco-2 cells, whereas HCT116 cells are provided with COX-1, but lack COX-2 expression (Figure 2c). Moreover, western blot analysis confirmed that in Caco-2 cells, the procedure of COX-2 mRNA silencing resulted in a marked reduction of COX-2 protein expression (Figure 2d).

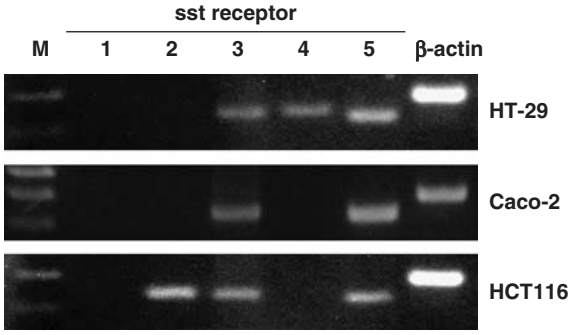

Figure 1 Reverse transcription (RT)–PCR analysis of mRNA encoding somatostatin receptor subtypes (sst_1, sst_2, sst_3, sst_4, sst_5) and β-actin in HT-29, Caco-2 and HCT116 cells. M, size markers.

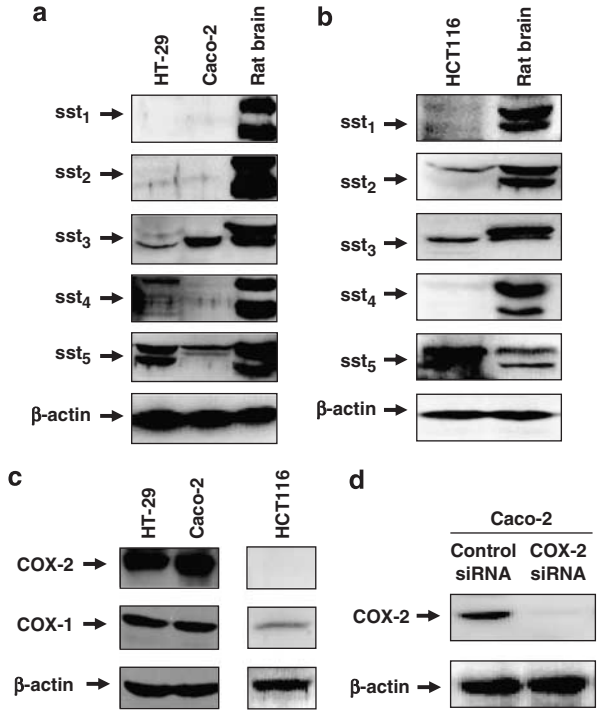

Figure 2 (**a**, **b**) Western blot analysis of somatostatin receptor subtypes (sst_1, sst_2, sst_3, sst_4, sst_5) and β-actin in HT-29, Caco-2, HCT116 cells and rat brain cortex. (**c**) Western blot analysis of cyclooxygenase (COX)-1, COX-2 and β-actin in HT-29, Caco-2 and HCT116 cells. (**d**) Western blot analysis of COX-2 expression in Caco-2 cells exposed to non-silencing control small-interfering RNA (siRNA) or COX-2 silencing siRNA oligonucleotides.

Studies under basal conditions

Effects of somatostatin-14 and somatostatin receptor ligands on cell growth, DNA synthesis and PGE_2 production. In preliminary experiments on Caco-2 cells, somatostatin-14 (0.1–10 nM) decreased cell growth in a concentration-dependent manner, with a maximal inhibitory effect at 1 nM (−68.7%). In these cells, basal DNA synthesis, as assessed by BrdU incorporation assay, was significantly reduced by somatostatin-14 (1 nM; −70.5%) and L-745337 (100 nM; −75.2%), a selective COX-2 blocker, but not modified by SC-560 (100 nM), a selective COX-1 blocker (Figure 3a). The inhibitory action of somatostatin-14 was counteracted by BN81658 (50 nM), a selective sst_3 receptor antagonist (Florio *et al.*, 2003), but was not affected by BIM23056 (50 nM), a selective sst_5 receptor antagonist (Wilkinson *et al.*, 1996; Figure 3a). In addition, basal DNA synthesis was inhibited by L-796778 (250 nM; −67.3%), a selective sst_3 receptor agonist (Rohrer, 1998), but not affected by BIM23206 (50 nM; −6.6%), a selective agonist of sst_5 receptors (Zatelli *et al.*, 2001). Exogenously applied PGE_2 (10 μM) enhanced DNA synthesis in Caco-2 cells when tested alone (+51.2%; $n=4$; $P<0.05$ versus control), and it fully reversed the inhibiting effect of somatostatin-14 1 nM (−3.8%; $n=4$; $P<0.05$ versus somatostatin-14 alone). When tested alone, BN81658 or BIM23056 did not affect basal DNA synthesis in Caco-2 cells (not shown).

Basal PGE_2 production in Caco-2 cells was significantly decreased by somatostatin-14 (1 nM) or L-745337 (100 nM), but it was not influenced by SC-560 (100 nM), and the inhibiting effect of somatostatin-14 was antagonized by BN81658 (50 nM), but not BIM23056 (50 nM; Figure 3b). BN81658 or BIM23056 did not affect PGE_2 production when assayed alone (not shown). Moreover, PGE_2 production in Caco-2 cells was reduced by L-796778 (250 nM; from 91 ± 10 pg 10^{-5} cells in controls to 37 ± 4 pg 10^{-5} cells, $n=5$, $P<0.05$), but not significantly affected by BIM23206 (50 nM; 89 ± 9 pg 10^{-5} cells, $n=5$). Following COX-2 mRNA silencing, Caco-2 cells displayed a marked decrease in DNA synthesis (−81.2%, $n=5$, $P<0.05$ versus control) and PGE_2 production (from 87 ± 9 pg 10^{-5} cells in controls to 23 ± 3 pg 10^{-5} cells, $n=5$, $P<0.05$), and both these parameters were not affected upon cell exposure to somatostatin-14 1 nM (−80.5% for DNA synthesis, $n=5$; 20 ± 4 pg 10^{-5} cells for PGE_2, $n=5$).

In HT-29 cells, somatostatin-14 (0.1–10 nM) did not exert appreciable effects on basal growth, DNA synthesis and PGE_2 production. In particular, upon exposure to somatostatin-14 10 nM, cell growth and DNA synthesis varied by −11.4% ($n=4$) and −14.3% ($n=4$), respectively, and PGE_2 release amounted to 69 ± 15 pg 10^{-5} cells ($n=4$; control value: 77 ± 13 pg 10^{-5} cells; $n=4$). Both DNA synthesis and PGE_2 production in HT-29 cells were not significantly influenced by L-796778 (250 nM; $n=4$), BIM23206 (40 nM; $n=4$) or L-803087 (10 nM; $n=4$), a selective agonist of SST4 receptors (Hannon *et al.*, 2002). In addition, PGE_2 production was almost suppressed by SC-560 (100 nM; 14 ± 4 pg 10^{-5} cells, $n=4$, $P<0.05$ versus control), but it was not significantly affected by L-745337 (100 nM; 64 ± 10 pg 10^{-5} cells, $n=4$).

When applied to HCT116 cells, which lack constitutive expression of COX-2, somatostatin-14 (0.1–10 nM) did not significantly affect basal growth, DNA synthesis and PGE_2 production. In particular, the variations of cell growth and DNA synthesis observed under somatostatin-14 10 nM were −7.8% ($n=5$) and −9.3% ($n=5$), respectively, and the PGE_2 release was 53 ± 10 pg 10^{-5} cells ($n=5$; control value: 58 ± 8 pg 10^{-5} cells, $n=5$). Moreover, PGE_2 production was decreased by SC-560 down to undetectable levels, whereas it was not significantly modified by L-745337 (100 nM; 56 ± 8 pg 10^{-5} cells, $n=5$).

Effects of somatostatin-14 and BN81658 on COX-2 expression. Western blot analysis on Caco-2 cells showed that somatostatin-14 (1 nM) decreased COX-2 expression,

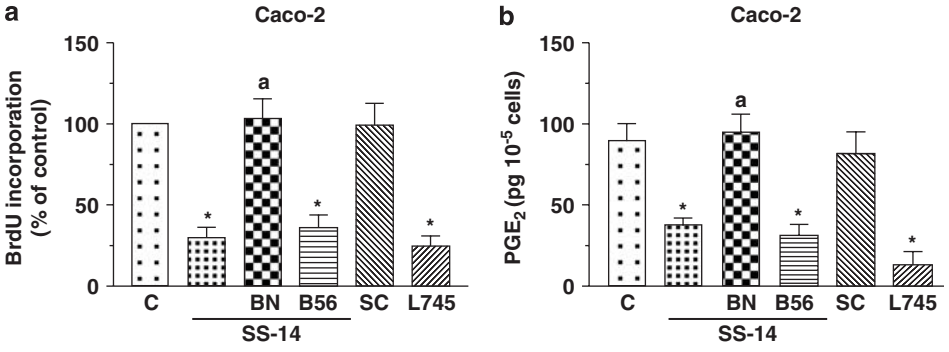

Figure 3 (**a**) Effects of somatostatin-14 (SS-14, 1 nM), SS-14 plus BN81658 (BN, 50 nM), SS-14 plus BIM23056 (B56, 50 nM), SC-560 (SC, 100 nM) or L-745337 (L745, 100 nM) on 5-bromo-2′-deoxyuridine (BrdU) incorporation in Caco-2 cells. (**b**) Effects of SS-14 (1 nM), SS-14 plus BN (50 nM), SS-14 plus B56 (50 nM), SC (100 nM) or L-745 (100 nM) on prostaglandin E_2 (PGE_2) release from Caco-2 cells. Each column represents the mean of 4–5 experiments ± s.e.mean (vertical bars). Significant difference from the respective control values (C), $^*P<0.05$; significant difference from SS-14 alone, $^aP<0.05$.

and that such inhibitory effects were antagonized by BN81658 (50 nM; Figure 4a). By contrast, somatostatin-14 did not significantly affect COX-2 expression in HT-29 cells when tested up to the concentration of 10 nM (Figure 4d).

Role of phosphotyrosine phosphatase and ERK-1/ERK-2 pathways in the regulatory actions of somatostatin-14 on COX-2 expression. In Caco-2 cells, the inhibiting action of somatostatin-14 (1 nM) on basal COX-2 expression was counteracted by orthovanadate (1000 nM), an inhibitor of phosphotyrosine phosphatase activity, but unaffected by GF109203X (1000 nM), a blocker of protein kinase C activity (Figure 4b). In addition, somatostatin-14 (1 nM) significantly reduced the phosphorylation status of ERK-1/ERK-2, and such an effect was reversed upon cell exposure to orthovanadate (Figure 4c).

Studies under stimulation with gastrin-17

Effects of somatostatin-14 and somatostatin receptor ligands on cell growth, DNA synthesis and PGE_2 production. The incubation of HT-29 cells with gastrin-17 (100 nM) was followed by significant increments in both cell growth (+53.3%) and PGE_2 release (+164.2%). Under these conditions, somatostatin-14 (0.1–10 nM) counteracted the growth-promoting effect of gastrin-17, and caused a concomitant reduction of PGE_2 production. In both cases, maximal inhibitory effects of somatostatin-14 were observed at the concentration of 1 nM (Figures 5a and b). In the presence of gastrin-17-induced stimulation, SC-560 (100 nM) did not affect cell growth and PGE_2 release. By contrast, both these functions were significantly inhibited by L-745337 (100 nM; Figures 5a and b). Gastrin-17 (100 nM) stimulated also cell DNA synthesis (+64.5%), and this response was associated with a significant increase in PGE_2 release. Somatostatin-14 (1 nM) counteracted the increment of DNA synthesis elicited by gastrin-17 and reduced PGE_2 production (Figures 5c and d). Both these inhibitory actions were mimicked by BIM23206 (50 nM), and reversed by blockade of sst_5 receptors with BIM23056 (50 nM; Figures 5c and d). Moreover, L-796778 (250 nM; $n=4$) or L-803087 (10 nM; $n=4$) did not significantly affect DNA synthesis (+61.6 and +67.3%, respectively) or PGE_2 release (+158.7 and +169.5%, respectively). In a set of additional experiments, exogenously applied PGE_2 (10 μM) did not further enhance gastrin-17-induced DNA synthesis in HT-29 cells when tested alone (+63.4%; $n=4$), but it fully reversed the inhibiting effect of somatostatin-14 1 nM (+67.1%; $n=4$; $P<0.05$ versus somatostatin-14 alone). When tested on Caco-2 or HCT116 cells, gastrin-17 (100 nM) did not significantly affect cell growth (+6.1 and +5.4%, respectively) or PGE_2 release (+4.7 and +6.8%, respectively).

Effects of somatostatin-14 and BIM23056 on COX-2 expression. Concentration-dependent inhibitory effects of somatostatin-14 (0.1–10 nM) on COX-2 expression were observed when HT-29 cells were stimulated with gastrin-17 (100 nM), and, in this setting, the modulating action of somatostatin-14 (1 nM) was antagonized by BIM23056 (50 nM; Figures 6a and b). By contrast, gastrin-17 (100 nM) failed to modify COX-2 expression in Caco-2 or HCT116 cells (not shown).

Role of phosphotyrosine phosphatase, protein kinase C and protein kinase G in the regulatory actions of somatostatin-14 on COX-2 expression. In HT-29 cells, the inhibitory effect exerted by somatostatin-14 (1 nM) on gastrin-17-evoked COX-2 induction was not influenced by inhibition of phosphotyrosine phosphatase with orthovanadate (1000 nM; Figure 6b). Under the same conditions, the stimulant effect of gastrin-17 on COX-2 expression was reversed by inhibition of protein kinase C with GF109203X (1000 nM), whereas it was not significantly modified by KT-5823 (500 nM), an inhibitor of protein kinase G (Figure 6c). COX-2 expression was enhanced also when HT-29 cells, in place of gastrin-17, were incubated with phorbol myristate acetate (100 nM), a direct stimulant of protein kinase C. However, this excitatory response was not affected by somatostatin-14 (1 nM; Figure 6d).

Effects of somatostatin-14 on activation of ERK-1/ERK-2 and Akt pathways. When applied to HT-29 cells, gastrin-17 (100 nM) enhanced the phosphorylation of kinases ERK-1/ERK-2 and Akt, belonging to MAPK and PI3K signalling pathways, respectively. Both these stimulant responses were

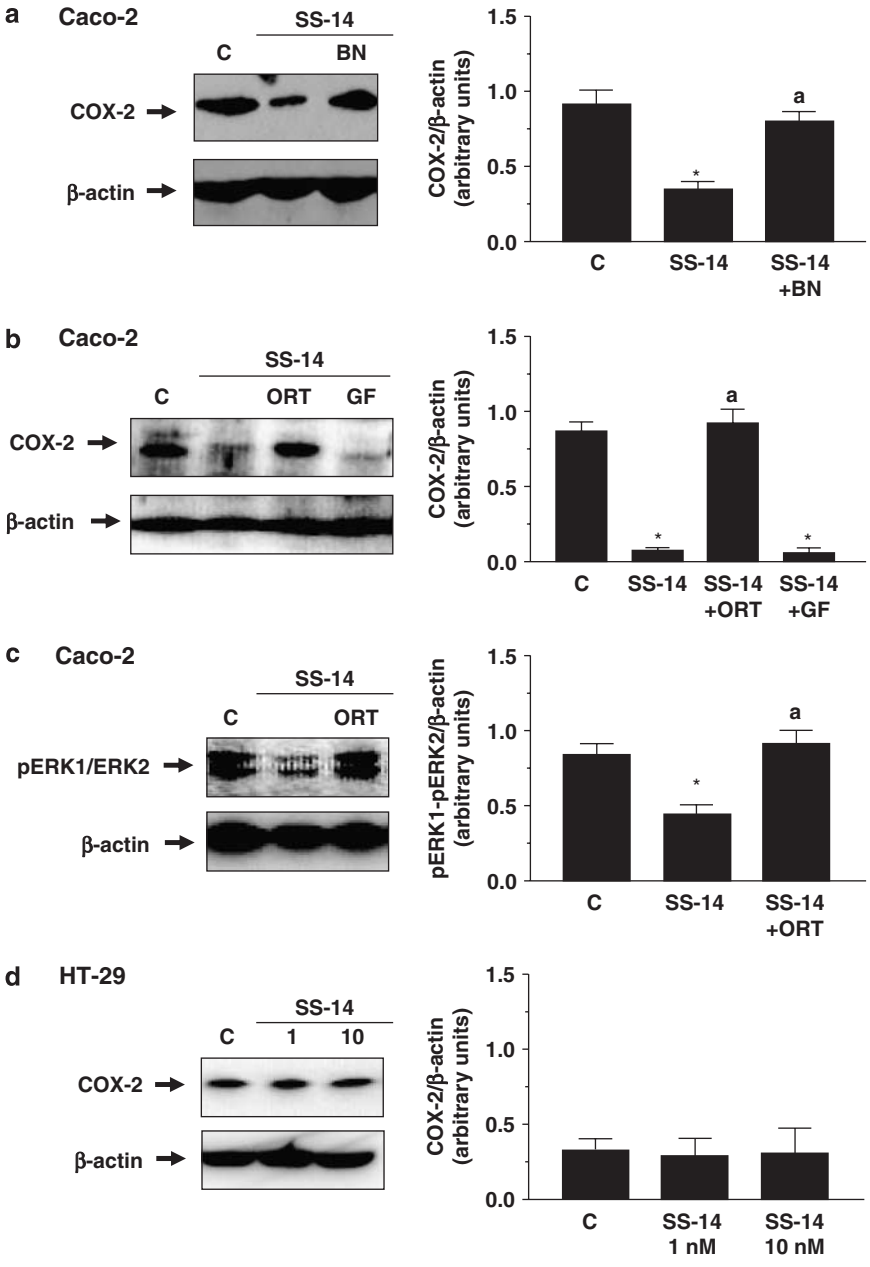

Figure 4 Western blot analysis in Caco-2 and HT-29 cells. (**a**) Effects of somatostatin-14 (SS-14, 1 nM), either alone or in the presence of BN81658 (BN, 50 nM), on cyclooxygenase-2 (COX-2) expression in Caco-2 cells. (**b**) Effects of SS-14 (1 nM), either alone or in the presence of orthovanadate (ORT, 1000 nM) or GF109203X (GF, 1000 nM), on COX-2 expression in Caco-2 cells. (**c**) Effects of SS-14 (1 nM), either alone or in the presence of ORT (1000 nM), on the phosphorylation of ERK-1/ERK-2 (pERK-1/ERK-2) in Caco-2 cells. (**d**) Effects of SS-14 (1–10 nM) on COX-2 expression in HT-29 cells. Column graphs refer to the densitometric analysis of COX-2 bands (**a**, **b**) or pERK-1/ERK-2 bands (**c**) normalized to the expression of β-actin. Each column represents the mean of 4–5 experiments ± s.e.mean (vertical bars). Significant difference from the respective control values (C), $^{*}P<0.05$; significant difference from values obtained in the presence of SS-14 alone, $^{a}P<0.05$.

counteracted by somatostatin-14 (0.1–10 nM) in a concentration-dependent manner (Figure 7).

Effects of somatostatin-14 on COX-2 expression, DNA synthesis and PGE_2 production under stimulation with epidermal growth factor. The application of EGF (100 ng mL^{-1}) to HT-29 cells resulted in a significant increase in COX-2 expression (Figure 8a). This stimulant action was associated with increments of both DNA synthesis and PGE_2 production (Figures 8b and c). Under these conditions, somatostatin-14 (1 nM) significantly inhibited the enhancing effects exerted by EGF on COX-2 expression, DNA synthesis and PGE_2 production (Figure 8).

Discussion

The observation of increased COX-2 expression in colorectal tumours, together with data indicating the ability of PGE_2 to stimulate colon cancer growth, has prompted

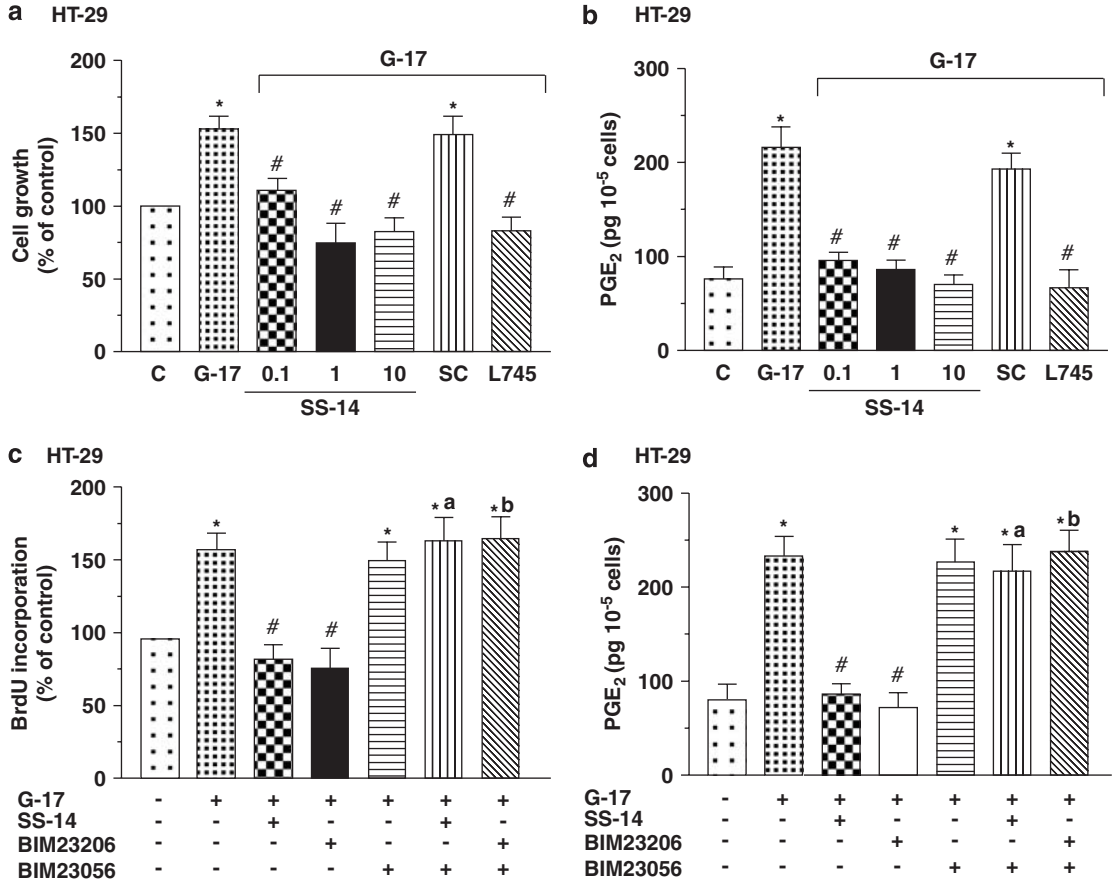

Figure 5 (Upper panels) Effects of somatostatin-14 (SS-14, 0.1–10 nM), SC-560 (SC, 100 nM) or L-745337 (L745, 100 nM) on HT-29 cell growth (**a**) and prostaglandin E_2 (PGE_2) release (**b**) under stimulation with gastrin-17 (G-17, 100 nM). (Lower panels) Effects of SS-14 (1 nM), BIM23206 (50 nM), BIM23056 (50 nM), SS-14 plus BIM23056 or BIM23206 plus BIM23056 on 5-bromo-2′-deoxyuridine (BrdU) incorporation (**c**) and PGE_2 release (**d**) in HT-29 cells under stimulation with G-17 (100 nM). Each column represents the mean of 5–6 experiments ± s.e.mean (vertical bars). Significant difference from the respective control values, $^{*}P<0.05$; significant difference from G-17 alone, $^{\#}P<0.05$. In panels (**c**) and (**d**), significant difference from SS-14, $^{a}P<0.05$ and from BIM23206, $^{b}P<0.05$.

clinical investigations on the use of COX-2 inhibitors for cancer prevention and treatment (Brown and DuBois, 2005; Chan *et al*., 2007). However, owing to serious concerns on safety of COX-2 inhibitors, the long-term use of these drugs in cancer patients is presently questioned (Bresalier *et al*., 2005), and therefore it appears of interest to identify alternative strategies to downregulate COX-2 expression/function at the level of colonic neoplasms, via pharmacological targeting of endogenous factors involved in the control of COX-2 pathway (Chun and Surh, 2004). The present study provides novel evidence indicating that somatostatin can reduce COX-2 expression and PGE_2 production in human colon cancer cells, and that such actions account for the ability of somatostatin to counteract cell proliferation.

Caco-2 and HT-29 cells were selected as models to examine mutual interactions between somatostatin and COX-2, as previous reports demonstrated the constitutive expression of COX-2 in these cell lines (Colucci *et al*., 2005; Tammali *et al*., 2006). In addition, Caco-2 cells subjected to COX-2 mRNA silencing as well as HCT116 cells, which lack COX-2 expression (Liu *et al*., 2003), were employed to assess the effects of somatostatin on cell models where the constitutive expression of COX-2 was absent or suppressed. In our study, the presence of sst receptor subtypes was detected in Caco-2 (sst_3, sst_5), HT-29 (sst_3, sst_4 and sst_5) and HCT116 cells (sst_2, sst_3 and sst_5) by both RT–PCR and western blot analysis. These findings are consistent with other investigations indicating the expression of multiple sst receptor subtypes in surgical specimens of human colon cancer. In particular, sst_2 receptors were shown to be almost equally expressed in normal and tumour tissues, with a trend towards receptor loss in malignant cells (Laws *et al*., 1997); the presence of sst_1, sst_3 and sst_4 receptor subtypes was found to be moderate in colon cancer cells (Laws *et al*., 1997; Vuaroqueaux *et al*., 2000), and sst_5 receptor expression was predominant in tumour cells over normal colonic mucosa (Vuaroqueaux *et al*., 1999, 2000). In our cell models, sst_3 receptor protein expression was predominant in Caco-2 cells, whereas the expression of sst_5 receptors predominated in HT-29 and HCT116 cells.

When assayed on Caco-2 cells, somatostatin-14 reduced the proliferative activity and COX-2 expression, and both these effects were proven to depend on stimulation of sst_3 receptors. Moreover, sst_3 receptor activation in this cell line was associated with a decrease in basal PGE_2 production.

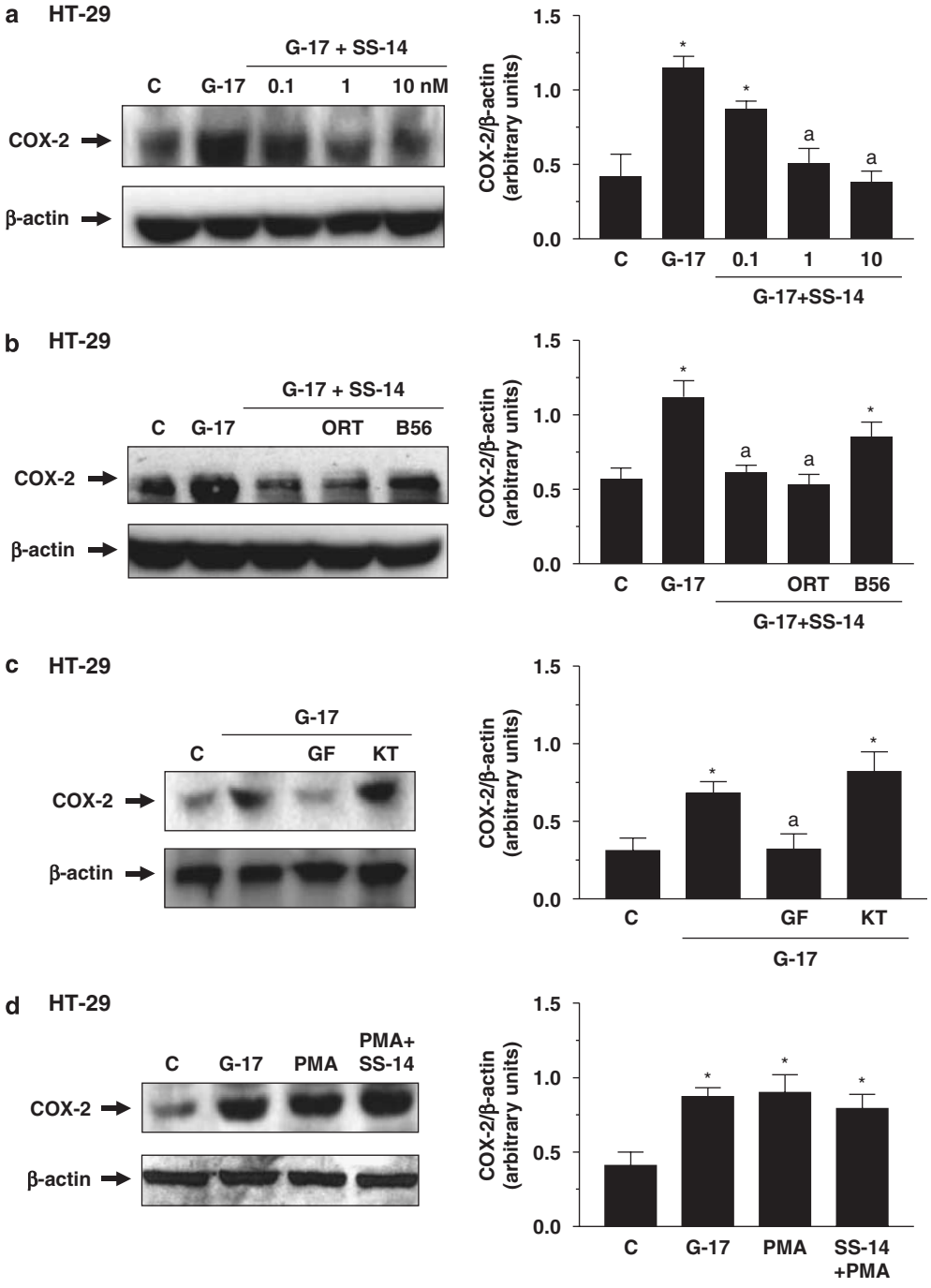

Figure 6 Western blot analysis showing the expression of cyclooxygenase-2 (COX-2) in HT-29 cells under the following conditions: (**a**) incubation with gastrin-17 (G-17, 100 nM), either alone or in the presence of increasing concentrations of somatostatin-14 (SS-14, 0.1–10 nM); (**b**) incubation with G-17 (100 nM), either alone or in the presence of SS-14 (1 nM), SS-14 plus orthovanadate (ORT, 1000 nM) or SS-14 plus BIM23056 (B56, 50 nM); (**c**) incubation with G-17 (100 nM), either alone or in the presence of GF109203X (GF, 1000 nM) or KT-5823 (KT, 500 nM); (**d**) incubation with G-17 (100 nM), phorbol myristate acetate (PMA, 100 nM) or PMA plus SS-14 (1 nM). Column graphs refer to the densitometric analysis of COX-2 bands normalized to the expression of β-actin. Each column represents the mean of four experiments ± s.e.mean (vertical bars). Significant difference from the respective control values (C), $*P<0.05$; significant difference from G-17 alone, $^{a}P<0.05$.

PGE_2, generated within colorectal neoplasms, can significantly enhance cell growth (Backlund *et al*., 2005; Castellone *et al*., 2005). In addition, sst_3 receptors can mediate anti-proliferative effects in cancer cells (Yoshitomi *et al*., 1997; Hu *et al*., 2004). Therefore, our findings, together with the observation that COX-2 blockade by L-745337 significantly inhibited DNA synthesis and PGE_2 release in Caco-2 cells and that exogenously applied PGE_2 counteracted the inhibiting action of somatostatin-14 on proliferation in this cell line, suggest that colon cancer cell growth can be reduced by somatostatin through downregulation of both COX-2 expression and function. This view is further

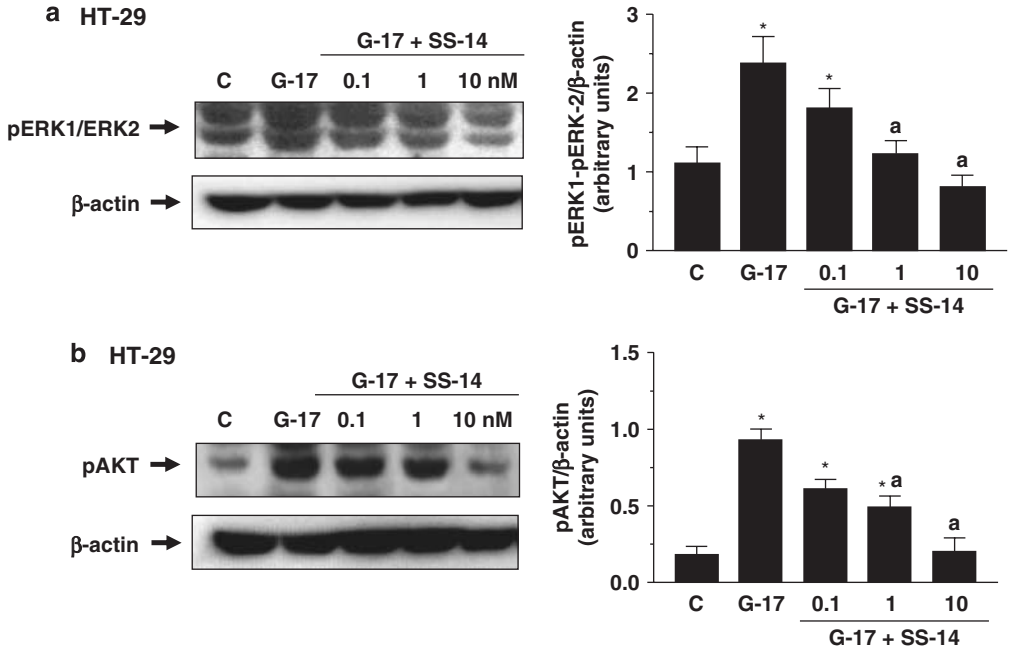

Figure 7 Western blot analysis showing the effects of increasing concentrations of somatostatin-14 (SS-14, 0.1–10 nM) on the phosphorylation of ERK-1/ERK-2 (pERK-1/ERK-2) (**a**) or Akt (pAkt) (**b**) in HT-29 cells under stimulation with gastrin-17 (G-17, 100 nM). Column graphs refer to the densitometric analysis of pERK-1/ERK-2 or pAkt bands normalized to the expression of β-actin. Each column represents the mean of 4–5 experiments ± s.e.mean (vertical bars). Significant differences from the respective control values (C), $*P<0.05$; significant differences from values obtained in the presence of G-17 alone, $^{a}P<0.05$.

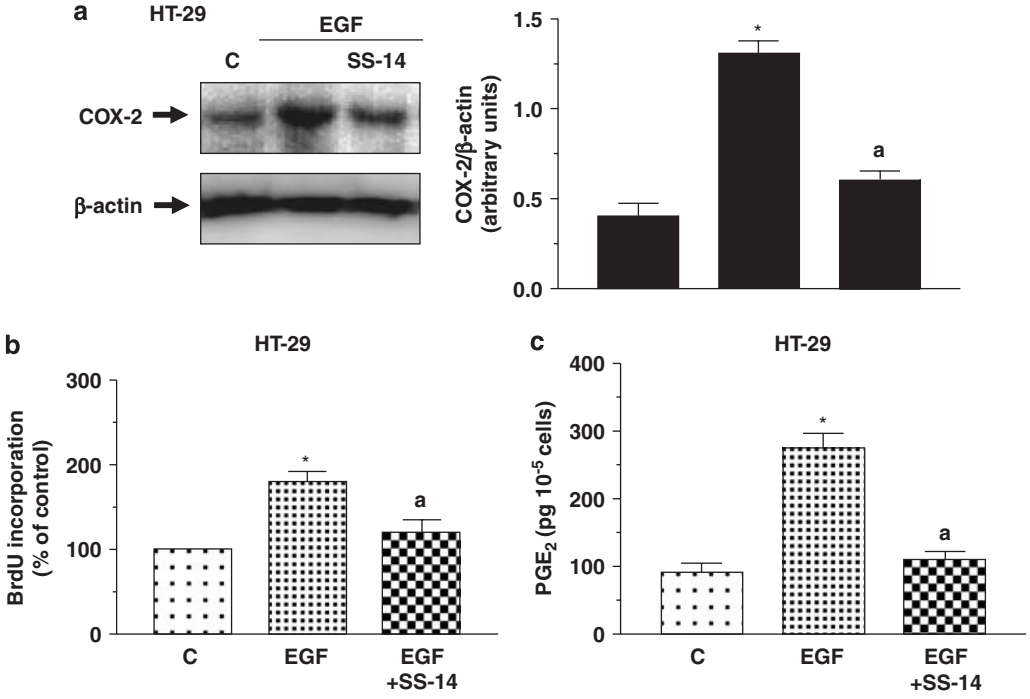

Figure 8 Effects of somatostatin-14 (SS-14, 1 nM) on cyclooxygenase-2 (COX-2) expression (**a**), 5-bromo-2′-deoxyuridine (BrdU) incorporation (**b**) and prostaglandin E_2 (PGE2) release (**c**) in HT-29 cells under stimulation with epidermal growth factor (EGF, 100 ng mL^{-1}). In (**a**), column graph refers to the densitometric analysis of COX-2 bands normalized to the expression of β-actin. Each column represents the mean of five experiments ± s.e.mean (vertical bars). Significant difference from the respective control values (C), $*P<0.05$; significant differences from values obtained in the presence of EGF alone, $^{a}P<0.05$.

supported by three additional observations made in the present study: (1) mRNA silencing of COX-2 in Caco-2 cells resulted in a marked reduction of PGE_2 production and DNA synthesis, and both these parameters were not affected further by somatostatin-14; (2) somatostatin-14 did not modify PGE_2 production and proliferation in HCT116 cells,

which lack constitutive COX-2 expression; (3) somatostatin-14 or agonists of sst_3, sst_4 and sst_5 receptors did not appreciably influence proliferation and PGE_2 production in HT-29 cells, which lack COX-2-dependent PGE_2 production. Overall, the differences in our results from Caco-2, HT-29 and HCT116 cells support the concept that the inhibiting actions mediated by sst_3 receptors on basal growth of colon cancer cells require a constitutive COX-2-dependent PGE_2 production to be fully effective.

The presence of gastrin/CCK_2 receptors in HT-29 cells (Colucci *et al*., 2005) offered us the opportunity to assay the effects of somatostatin-14 under stimulation of this cell line with gastrin-17. In this setting, somatostatin-14 was able to inhibit cell growth and DNA synthesis as well as COX-2 expression in HT-29 cells, and such modulating actions were associated with a concomitant decrease in gastrin-17-induced PGE_2 release. As in our experiments L-745337 counteracted the stimulant effects of gastrin-17 on cell proliferation and PGE_2 production, and exogenously applied PGE_2 reversed the decreasing effect of somatostatin-14 on DNA synthesis, it can be proposed that COX-2 inhibition plays a causal role in the growth inhibiting action exerted by somatostatin-14 in the presence of gastrin-17-induced stimulation. In the attempt to characterize the sst receptor pathways implicated in these regulatory actions, we observed that the inhibitory effects of somatostatin-14 on gastrin-17-induced proliferation and PGE_2 release were mimicked by the sst_5 agonist BIM23206, but not by sst_3 or sst_4 agonists, and that the modulating effects of somatostatin-14 or BIM23206 on the evoked cell growth, COX-2 induction and PGE_2 production were abolished by the sst_5 antagonist BIM23056. These findings point out the novel concept that somatostatin has the potential to counteract the growth-promoting effects of gastrin on colonic tumours, and that sst_5 receptors, acting via a decrease in COX-2-dependent PGE_2 biosynthesis, play a prominent role in this regulatory action. In addition, as somatostatin-14 exerted similar inhibitory effects when HT-29 cells were exposed to EGF, it appears that somatostatin may retain the potential to modulate COX-2-dependent proliferation of colon cancer cells also under stimulation with growth factors other than gastrin.

It is presently recognized that sst receptor subtypes can recruit distinct signalling pathways to regulate cellular functions (Moller *et al*., 2003). With regard for cell growth, the modulating actions of somatostatin appear to be primarily mediated by phosphotyrosine phosphatase (Reardon *et al*., 1997; Florio *et al*., 2001), and the ability to activate this pathway has been demonstrated for sst_3 and sst_5 receptors (Reardon *et al*., 1997; Moller *et al*., 2003). In addition, sst_5 receptors can arrest cell proliferation through inhibitory coupling to phospholipase C/inositol phospholipids pathway (Buscail *et al*., 1995), as well as by inhibition of MAPK pathway via negative regulation of guanylyl cyclase and cGMP-dependent protein kinase G (Cordelier *et al*., 1997). In the present study, the decrease in COX-2 expression caused by somatostatin-14 in Caco-2 cells was reversed by blockade of phosphotyrosine phosphatase with orthovanadate, whereas the protein kinase C inhibitor GF109203X did not exert any influence. In addition, the phosphorylation status of ERK-1/ERK-2 was reduced after cell incubation with somatostatin-14, and such effects were prevented by orthovanadate. These results suggest that somatostatin can attenuate the constitutive expression of COX-2 in colon cancer cells through receptor-mediated activation of phosphotyrosine phosphatase, leading to inhibition of the MAPK pathway. In keeping with this view, sst_3 receptors were shown to inhibit MAPK pathway via phosphotyrosine phosphatase activation (Reardon *et al*., 1996; Yoshitomi *et al*., 1997), and the blockade of phosphotyrosine phosphatase was reported to upregulate COX-2 (Barat and Tremblay, 2003).

With regard for gastrin-17-induced COX-2 upregulation in HT-29 cells, we considered that gastrin promotes cell growth by recruitment of phospholipase C/protein kinase C-dependent signals, causing downstream activation of MAPK and PI3K/Akt pathways (Rozengurt and Walsh, 2001). Moreover, gastrin was shown to generate MAPK- and PI3K-mediated signals, which target the COX-2 gene promoter to upregulate COX-2 expression and induce an increase in PGE_2 release (Colucci *et al*., 2005). In the present study, the stimulant action of gastrin-17 on COX-2 expression was reversed by blockade of protein kinase C, but was not affected by KT-5823, a blocker of protein kinase G. Moreover, the inhibitory effects of somatostatin-14 on gastrin-17-induced COX-2 expression were not modified by inhibition of phosphotyrosine phosphatase, and they no longer occurred when cells were exposed to phorbol myristate acetate, a direct stimulant of protein kinase C. These findings, taken together with data from our additional experiments, showing that gastrin-17-induced phosphorylations of ERK-1/ERK-2 and Akt were counteracted by somatostatin-14, support the notion that somatostatin can oppose proliferative stimuli via downregulation of COX-2 expression driven by a negative regulation of protein kinase C-dependent MAPK and Akt activation.

When attempting to translate the present findings to clinical settings, it should be considered that studies with somatostatin analogues in patients with advanced colorectal cancer have failed to demonstrate significant benefits (Goldberg *et al*., 1995), likely as a consequence of a loss of sst receptor expression in patients with lymph node or distant metastases, thus suggesting a possible efficacy of somatostatin only in a limited number of patients with tumours expressing specific sst receptor subtypes. Previous studies have reported a predominance of sst_5 receptor expression in colorectal cancer tissues, in concomitance with a moderate expression of sst_3 receptors (Vuaroqueaux *et al*., 1999). However, more recent data suggest that all five sst receptor subtypes are involved in colorectal cancer growth and, in particular, that cancer cells lacking sst_3 receptor expression display an enhanced proliferative activity (Qiu *et al*., 2006). Therefore, based on these observations and our findings, indicating a COX-2-dependent control of sst_3 and sst_5 receptors on colon cancer cell growth, it is conceivable that somatostatin analogues, endowed with a wide spectrum of agonistic activity on sst receptor subtypes, might display an improved therapeutic efficacy against colorectal cancer, particularly in the presence of cancer tissues with constitutive or stimulated COX-2 expression.

The role of gastrin in human colorectal cancer is also greatly debated, mainly because its receptors have been found in a low proportion (about 40%) of tumour specimens examined (Biagini *et al*., 1997; Schmitz *et al*., 2001), and therefore gastrin stimulation might be regarded as a infrequent condition in colorectal cancer patients. On the other hand, prolonged hypergastrinaemia has been recognized as a risk factor for colorectal cancer (Thorburn *et al*., 1998) and, in the present study, the inhibiting activity of somatostatin-14 on COX-2-dependent cell proliferation was observed not only against gastrin-17, but also under stimulation with EGF, thus suggesting that the modulation of COX-2 expression/activity may downregulate colon cancer growth under different pathophysiological settings. Nevertheless, it remains to be clarified whether, and to what extent, our *in vitro* results, on the relationships between somatostatin receptor signalling, COX-2 function and cell growth, can be relevant to the *in vivo* proliferation of colorectal cancers, and studies on animal models might contribute to properly address this issue.

In conclusion, the present study provides novel evidence that somatostatin can decrease COX-2 expression and enzyme function in human colon cancer cells via activation of sst_3 or sst_5 receptor subtypes, and suggests that these effects contribute significantly to the inhibitory action of somatostatin on cell growth.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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