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Bombesin induces cyclooxygenase-2 expression through the activation of the nuclear factor of activated T cells and enhances cell migration in Caco-2 colon carcinoma cells

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Cyclooxygenase-2 (Cox-2), the gastrin-release peptide (GRP) and its cognate receptor (GRP-R) are overexpressed in a significant percentage of colorectal carcinomas and are associated with cell growth, invasiveness and tumor progression. However, a molecular link between all of them in adenocarcinomas has not been established. Here, we show that bombesin (BBS), a GRP homolog, stimulates the expression of Cox-2 mRNA and protein in human colon adenocarcinoma Caco-2 cells, resulting in enhanced release of prostaglandin E_2. These effects were markedly inhibited by the specific BBS antagonist RC-3940-II. BBS promotes the activation of the nuclear factor of activated T cells (NFAT) through a Ca^{2+}/calcineurin (Cn)-linked pathway. Upon BBS stimulation, the NFATc1 isoform translocates into the nucleus with a concomitant increase in NFATc1 binding to two specific recognition sites in the promoter region of the Cox-2 gene. Furthermore, inhibition of Cn activity by the immunosuppressive drug cyclosporin A impaired NFAT activation and diminished Cox-2 expression in BBS-stimulated cells. Interestingly, BBS pretreatment strongly enhances the invasive capacity of carcinoma cells, effect which was inhibited by a Cox-2-specific inhibitor. These findings provide the first evidence for the involvement of the Ca^{2+}/Cn/NFAT pathway in BBS-mediated induction of genes involved in colon carcinoma invasiveness such as Cox-2.

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Keywords: cyclooxygenase-2; NFAT; bombesin; migration; colon carcinoma

Introduction

Cyclooxygenases (Cox-1 and Cox-2) are considered to play an important role in human colon cancer, one of the major causes of mortality in developed countries (Kawai *et al*., 2002). These enzymes catalyse the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. Cox-1 isoform is constitutively expressed in many human tissues. In contrast, Cox-2 is present at very low levels under basal conditions, but is rapidly induced in response to a panel of inflammatory, mitogenic and pro-oncogenic stimuli in a wide variety of cell types, including cancer cells (reviewed by Smith *et al*., 2000). Considerable interest is currently focused on Cox-2 as a potential therapeutic target for the prevention and treatment of cancer (Kawai *et al*., 2002; Iniguez *et al*., 2003). Numerous studies indicate that Cox-2 is overexpressed in 85–90% of cancerous tissues from human colon and inhibition of Cox-2 by non-steroidal anti-inflammatory drugs decreases the risk of colorectal cancer (revised by Gupta and Dubois, 2001; Chan, 2002). Experiments with *Apc* gene-deficient mice (Min mice) and $Apc^{\Delta 716}$/*Cox-2* double-knockout mice revealed that either pharmacologic inhibition or genetic ablation of Cox-2 resulted in reduced intestinal tumorigenesis (Oshima *et al*., 1996; Williams *et al*., 1996). Enhanced synthesis of Cox-2-derived PGs may favor tumor growth by stimulating cell proliferation, promoting angiogenesis, inducing local immunosuppression, increasing invasiveness and inhibiting apoptosis (Kawai *et al*., 2002; Iniguez *et al*., 2003). Nevertheless, Cox-2-specific inhibitors may significantly increase cardiovascular risk (Bresalier *et al*., 2005) and some clinical trials indicate that the Cox-2 inhibitor Rofecoxib has little effect on proliferation or apoptosis of colorectal cancer liver metastases (Fenwick *et al*., 2003).

The mechanisms leading to enhanced Cox-2 expression in intestinal epithelial cells have not been fully elucidated. Both transcriptional and post-transcriptional mechanisms regulate Cox-2 expression in colon carcinoma (Shao *et al*., 2000; Cheng and Harris, 2002; Inoue *et al*., 2002; Ramsay *et al*., 2003; Dixon, 2004). Moreover, depending on the stimuli, multiple signaling pathways including mitogen-activated protein kinases extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase and p38 have been shown to mediate Cox-2 induction in human colon cancer cells (Guo *et al*., 2001; Liu *et al*., 2003; Shao *et al*., 2004; Sun and Sinicrope, 2005).

On the other hand, nuclear factor of activated T cells (NFAT) is required for optimal induction of Cox-2 promoter activity in a diversity of cell types (Robida

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et al., 2000; Iniguez *et al*., 2000; Hernandez *et al*., 2001; Lara-Pezzi *et al*., 2002; Duque *et al*., 2005). The NFAT family includes four 'classical' members that display a high degree of homology: NFATc2 (also called NFAT1 or NFATp), NFATc1 (2 or c), NFATc3 (4) and NFATc4 (Hogan *et al*., 2003). NFAT exists in a highly phosphorylated form in the cytoplasm, which translocates into the nucleus upon dephosphorylation by the phosphatase calcineurin (Cn) in response to increases in intracellular calcium ($[Ca^{2+}]_i$), where it binds to enhancer elements of specific genes leading to transcriptional activation. Cn activity can be blocked by cyclosporin A (CsA), thereby preventing the nuclear translocation of NFAT (Hogan *et al*., 2003). Although many stimuli have been demonstrated to be able to induce this signaling pathway in non-immune cells (Hogan *et al*., 2003), the physiological agents that lead to the activation of NFAT in tumor cells have not been established.

Recent studies have described that bombesin (BBS), a homologue of the mammalian gastrin-release peptide (GRP), induces Cox-2 expression in fibroblasts and epithelial cells, requiring an increase in $[Ca^{2+}]_i$ (Hecht *et al*., 1997; Guo *et al*., 2001). Even more, GRP and its cognate G-protein-coupled receptor (GRP-R), as well as Cox-2, are overexpressed in a substantial fraction of human colorectal carcinomas (38–76%) (Preston *et al*., 1995; Carroll *et al*., 1999, 2000; Gupta and Dubois, 2001; Jensen *et al*., 2001). BBS/GRP are neurotransmitters expressed mainly in nerve fibers throughout the mammalian gut and in the central nervous system. In addition to their physiological neuroendocrine actions, BBS/GRP have growth-promoting activity in various cancers, including pancreatic, prostate, mammary and small-cell lung carcinomas, which may secrete BBS/GRP in an autocrine manner (Cuttitta *et al*., 1985). Conversely, BBS/GRP antagonists can effectively inhibit tumor growth and slow the progression of premalignant lesions (Iishi *et al*., 2003; Kanashiro *et al*., 2003; Yegen, 2003). The abnormal expression of BBS/GRP and GRP-R in human colon cancers has been correlated with more invasive characteristics of these tumors. Several reports show that BBS induces morphological and adhesive, rather than proliferative, effects on human colon carcinoma cells, suggesting that expression of BBS/GRP-R may regulate cell differentiation and thus contribute to their malignant properties (Saurin *et al*., 1999, 2002; Jensen *et al*., 2001).

Altogether, these observations prompted us to examine the regulation of Cox-2 expression by BBS in human colon adenocarcinoma cell lines. Our results show that induction of Cox-2 expression by BBS in these cells requires activation of the Ca^{2+}/Cn/NFAT pathway. NFAT is translocated to the nucleus upon BBS stimulation where it binds to NFAT response elements in the promoter region of Cox-2 gene that are essential for Cox-2 transcriptional induction. Moreover, BBS through Cox-2 induction promotes migration of colon carcinoma cells. These findings demonstrate the participation of NFAT in BBS-mediated induction of genes involved in colon carcinoma invasiveness such as Cox-2.

Results

BBS stimulates Cox-2 expression and PGE_2 production

BBS induced a dose-dependent increase in the expression of Cox-2 mRNA in serum-starved Caco-2 cells, reaching maximum levels in cells treated with 100 and 1000 nM BBS, although some effect can be seen at 10 nM (Figure 1a). Kinetics experiments showed maximal induction of Cox-2 after 4 h incubation in the presence of BBS (Figure 1b). Analysis of Cox-2 protein levels by Western blot also revealed a time-dependent increase that was clearly observed at 4 h following BBS (100 nM) stimulation, with the highest expression between 8 and 24 h (Figure 1c). Interestingly, Cox-2 protein induction was markedly inhibited by RC-3940-II, a well-known BBS/GRP-R antagonist (Cai *et al*., 1994), indicating the specificity of BBS action (Figure 1c). Expression of the non-inducible Cox-1 isoform was almost negligible, and was not affected by BBS treatment (not shown).

To assess whether induction of Cox-2 expression was associated with an increase in enzymatic activity, prostaglandin E_2 (PGE_2) release by Caco-2 cells was measured. BBS (100 nM) induced a significant time-dependent release of PGE_2 to the media compared to untreated cells over a period of 24 h ($P<0.001$) (Figure 1d). PGE_2 levels were drastically reduced in the supernatant from cells incubated in the presence of a Cox-2-selective inhibitor (NS398 1 μM) ($P<0.02$), further confirming the involvement of Cox-2 in the increase of PGE_2 production upon BBS stimulation. Similar induction was observed in other carcinoma cell lines as HT-29 (data not shown).

Involvement of Ca^{2+}/Cn signaling pathway in BBS-mediated upregulation of Cox-2 expression

Agonist binding to GRP-R results in the rapid release of Ca^{2+} from inositol 1,4,5-triphosphate-sensitive stores (Hellmich *et al*., 1999). To test whether BBS induced an increase in $[Ca^{2+}]_i$, Caco-2 cells were loaded with the Ca^{2+} indicator dye Fura-2/AM and stimulated with BBS (100 nM) in the presence or absence of the BBS antagonist RC-3940-II. BBS elicited a rapid increase in $[Ca^{2+}]_i$, which was blocked by pretreating the cells with RC-3940-II (10 nM) (Figure 2a), confirming the expression of functional BBS/GRP-R on these cells (Carroll *et al*., 2000). This augmentation in $[Ca^{2+}]_i$ could mediate activation of Cn, thus leading to NFAT activation and subsequently to Cox-2 induction. As shown in Figure 2b, inhibition of Cn activity by CsA treatment diminished Cox-2 protein expression induced by BBS. Accordingly, CsA-mediated inhibition of Cox-2 expression results in a significant ($P<0.05$) reduction in BBS-induced PGE_2 production (Figure 1d). Taken together, these data suggest that BBS upregulation of Cox-2 expression in Caco-2 cells requires an increase in $[Ca^{2+}]_i$ and a subsequent Cn activation.

BBS induces nuclear translocation of NFAT and its binding to DNA

We next investigated whether NFAT, a Ca^{2+}/Cn-regulated transcription factor, participates in Cox-2

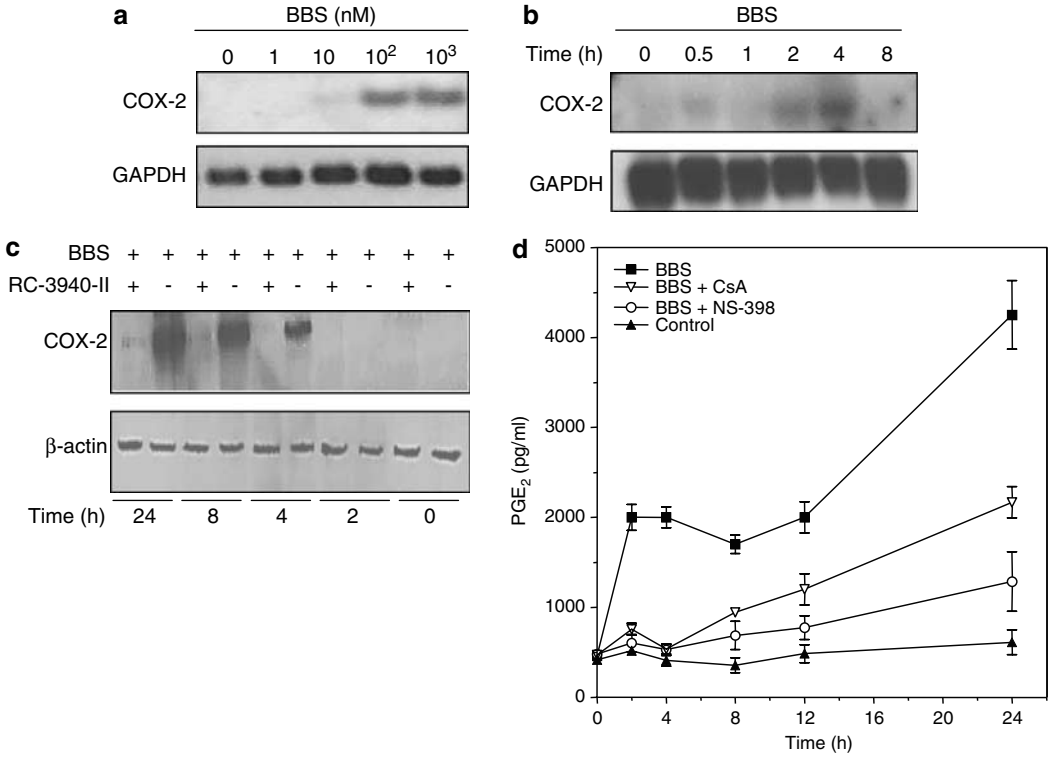

Figure 1 BBS increases Cox-2 expression and PGE_2 production in Caco-2 cells. (**a**) Dose effect of BBS on Cox-2 mRNA expression. Serum-starved Caco-2 cells were treated with the indicated concentration of BBS for 4 h, and the levels of Cox-2 mRNA were assessed by Northern blot. GADPH levels were measured as a control of loading. (**b**) Time course of Cox-2 mRNA expression. Caco-2 cells were treated with BBS (100 nM) for the indicated times (hours) and mRNA levels of Cox-2 and GAPDH determined by Northern blot. (**c**) Time course of Cox-2 protein expression. Caco-2 cells were incubated with BBS (100 nM), in the presence or in the absence of the specific BBS antagonist RC-3940-II (10 nM) for the indicated times, and Cox-2 and β-actin proteins were analysed by Western blotting as described under 'Materials and methods'. (**d**) Induction of PGE_2 synthesis by BBS. Caco-2 cells were pretreated or not with 1 μM NS398 or 200 ng/ml CsA for 2 h and subsequently treated with or without BBS (100 nM) for an additional 24 h. PGE_2 levels were determined by enzyme immunoassay in the supernatants.

induction by BBS. Our results showed that NFATc1 is present in the cytoplasm of untreated Caco-2 cells and is rapidly (30 min) dephosphorylated upon BBS stimulation and translocated into the nucleus (Figure 3a). Pretreatment with CsA (1 μg/ml) inhibited NFAT translocation, thereby resulting in an accumulation of cytoplasmic NFATc1 protein.

To analyse DNA binding of BBS-activated NFAT to the distal NFAT (dNFAT) and proximal NFAT (pNFAT) sequences of the human Cox-2 promoter, we performed electrophoretic mobility shift assays (EMSAs) with nuclear extracts of Caco-2 cells treated with BBS (100 nM) or calcium ionophore A23187 (Ion, 1 μM) as a control, using oligonucleotides from pNFAT or dNFAT sites of Cox-2 promoter (Iniguez *et al*., 2000) (Figure 3b). Both the pNFAT and dNFAT Cox-2 probes specifically bound nuclear proteins from Caco-2 cells. The retarded complexes were efficiently competed with a 20-fold molar excess of the respective cold oligonucleotides. Although some constitutive binding was observed in unstimulated cells, especially to the pNFAT probe, BBS induced an enhanced binding to the same level than Ion. Furthermore, these inducible complexes were severely diminished in nuclear extracts from cells stimulated with BBS in the presence of CsA. To determine unambiguously the presence of the NFATc1 protein in the bands, we performed EMSAs using an anti-NFATc1 polyclonal antiserum, which clearly prevented the binding of specific complexes allowing the formation of more retarded complexes likely constituted by DNA/NFAT/antibody. As a control, EMSAs were carried out in the presence of preimmune serum (Figure 3b). These data suggest that the binding of NFATc1 protein to the Cox-2 dNFAT and pNFAT sites in response to BBS treatment of Caco-2 cells.

Transcriptional regulation of the Cox-2 promoter by BBS

We next analysed whether the effect of BBS on Cox-2 expression was taking place at the transcriptional level. For this, Caco-2 cells were transfected with a Cox-2 promoter/luciferase construct spanning from nucleotide -1796 to $+104$ bp relative to the human Cox-2 gene transcription start site (P2-1900-Cox-2-LUC) (Iniguez *et al*., 2000). As shown in Figure 4, BBS (100 nM) induced a fourfold increase ($P<0.001$) in luciferase activity in transiently transfected cells compared to untreated controls. CsA treatment significantly inhibited BBS-mediated induction of Cox-2 promoter.

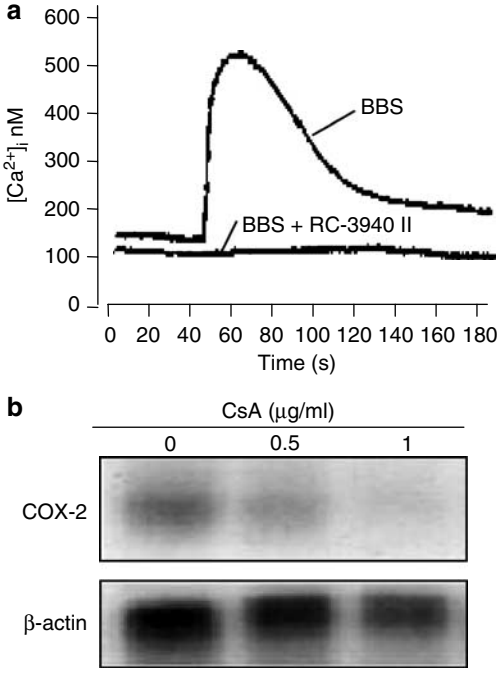

Figure 2 BBS-mediated upregulation of Cox-2 expression requires Cn activation. (**a**) Caco-2 cells were loaded with the Ca^{2+} indicator dye Fura-2/AM and changes in $[Ca^{2+}]_i$ upon BBS treatment were recorded in the presence or absence of the BBS/GRP-R selective antagonist RC-3940-II. (**b**) Effect of CsA on Cox-2 protein expression. Protein extracts from Caco-2 cells were treated or not with the indicated concentration of CsA 30 min before BBS stimulation for 6 h and analysed by Western blotting. β-Actin protein levels were determined as a control of loading.

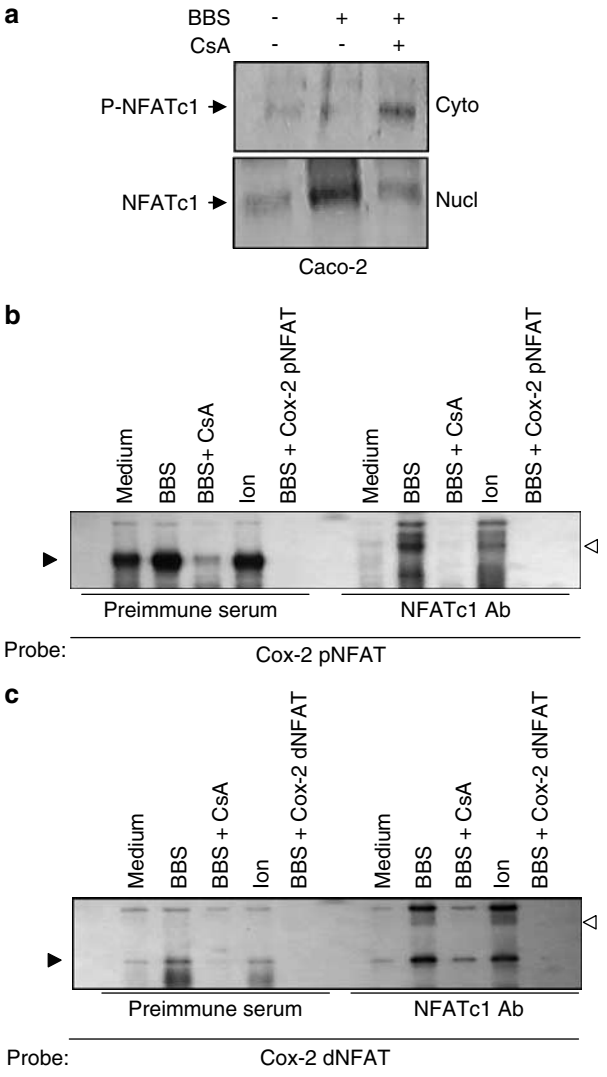

Figure 3 BBS induces NFAT activation in Caco-2 cells. (**a**) Activation of NFATc1 in Caco-2 colon carcinoma cells. Caco-2 cells were incubated for 30 min with BBS (100 nM). CsA (1 μg/ml) was added 30 min before stimulation. Fractionated extracts from both untreated and treated cells were analysed by Western blotting with an antiserum to NFATc1. Arrows indicate the phosphorylated cytosolic or dephosphorylated nuclear forms of NFATc1. Cyto, cytosolic extracts; Nucl, nuclear extracts. (**b**) EMSA analysis of NFATc1 binding to the pNFAT and dNFAT sites of the human Cox-2 gene (Cox-2 pNFAT and dNFAT, respectively). Caco-2 cells were incubated with medium, BBS in the presence or absence of CsA or with calcium Ion (0.5 μM) for 30 min as indicated. Nuclear extracts were analysed by EMSA using Cox-2 pNFAT (upper panel) or Cox-2 dNFAT (lower panel) radiolabeled probes. A 50-fold molar excess of unlabeled Cox-2 pNFAT and dNFAT oligonucleotides (BBS + Cox-2 pNFAT and BBS + Cox-2 dNFAT, respectively) was added to determine specific binding. The NFATc1 antiserum (NFATc1 Ab) or preimmune serum was added to the extracts before incubation with the probes. Closed arrows indicate specific complexes. White arrows indicate supershifted complexes.

To map the region responsible for BBS inducibility, we used Cox-2 promoter deletion constructs. BBS significantly induced transcription driven by constructs with deletions up to −88 of the Cox-2 promoter region (P2-1900 to P2-192), although a statistically significant decrease in inducibility was observed in P2-192 construct that contains the pNFAT site (Figure 5a). However, deletion of *cis*-acting elements mapping between the nucleotides at positions −88 and −46 in the promoter region completely abolished the BBS-mediated increase of luciferase activity.

Given the relevance of the region spanning from nucleotides −170 to −46 for the induction of the Cox-2 promoter by BBS, we next determined the contribution of the two NFAT sites present in this region to the overall transcriptional regulation of the Cox-2 promoter by BBS by using the P2-274 containing specific mutations within the dNFAT site, the pNFAT site or both (Iniguez *et al*., 2000). Transfection experiments showed that mutation of the dNFAT (P2-274 dNFAT mut) or pNFAT (P2-274 pNFAT mut) sites resulted in a 29 and a 49% loss in the BBS-induced Cox-2 promoter activity, respectively, whereas double mutation of both NFAT (P2-274 p- and dNFAT mut) sites drastically reduced the activation of the promoter by BBS. Mutagenesis of the AP-1-like site (P2-274 AP-1 mut) also affected the inducibility of the Cox-2 promoter by BBS, reflecting a possible contribution of AP-1 to BBS-mediated Cox-2 induction (Figure 5b).

To further confirm the involvement of NFAT activation in the transcriptional regulation mediated by BBS in Caco-2 cells, we co-transfected expression

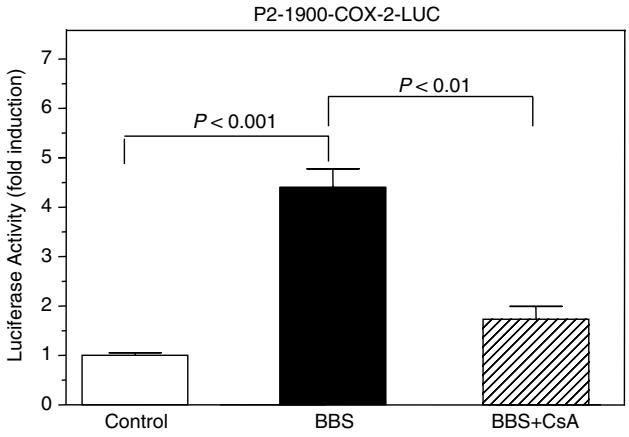

Figure 4 Effect of CsA on the BBS-mediated inducibility of the Cox-2 promoter. Caco-2 cells were transiently transfected with the P2-1900-Cox-2-LUC reporter construct and stimulated with BBS (100 nM) in the presence or absence of CsA (100 ng/ml) for 16 h. Luciferase activity is expressed as fold induction relative to the transfection with empty expression vector. Data are the means ± s.e. of three independent experiments, each performed in triplicate. Differences between BBS and BBS + CsA, and between BBS and the control were significant at $P<0.01$ and $P<0.001$, respectively.

vectors, encoding either a functional NFATc2 protein (hemagglutinin (HA)-NFAT) or a dominant-negative version of NFATc1 (dnNFAT), together with the P2-274-Cox-2-LUC or the NFAT-LUC reporter plasmids. BBS treatment was able to activate both reporters and to augment the induction mediated by overexpression of HA-NFAT. More interestingly, expression of dnNFAT, previously described to abolish NFAT-dependent promoter activity (Iniguez *et al*., 2000), abrogated BBS-induced transcription of both reporters (Figure 6a and b), further supporting the hypothesis of the involvement of NFAT signaling in the regulation of Cox-2 gene expression by BBS. A similar pattern was observed using phorbol 12-myristate 13-acetate (PMA) + Ion as stimuli for both NFAT-LUC and P2-274-Cox-2-LUC reporters (Figure 6c and d).

BBS stimulates Caco-2 cells invasiveness

Previous results have described the ability of BBS to alter the morphology of colon carcinoma cells increasing tumor invasiveness (Saurin *et al*., 1999, 2002). Thus, we investigated whether this kind of modifications effectively occurred in BBS-treated Caco-2 cells, largely reputed as poorly aggressive (Engle *et al*., 1998). For this, we used a Matrigel Chamber invasion assay. Under our experimental conditions, this cell type was not able to pass through the Matrigel + membrane barrier when fetal calf serum (FCS)-free Dulbecco's modified Eagle's medium (DMEM) was introduced into the lower compartment. There was a small number of invading cells when FCS-supplemented medium was used in place of serum-free DMEM (Figure 7a). As reported previously (Kermorgant *et al*., 2001), when 100 ng/ml of hepatocyte growth factor (HGF) were added to the FCS-DMEM, there was a substantial increase in the number of invading cells. BBS addition to either FCS-free or FCS-supplemented medium slightly induced (around twofold) the migration of Caco-2 cells. Interestingly, when Caco cells were pretreated for 3 days in the presence of 100 nM BBS and then subjected to *in vitro* invasion assay, a significant ($P<0.05$) increase in cell invasiveness could be observed, independently from the chemoattractants (e.g. FCS-supplemented DMEM with or without BBS or HGF) dispensed into the lower chamber (Figure 7b). The MDA-MB-231 human breast carcinoma cell line, showing marked invasion capacity in Transwell assays (Munoz-Najar *et al*., 2006) was used as a positive control. More interestingly, the enhancing effect of BBS treatment was completely abolished by addition of the BBS receptor antagonist RC-3940-II (10 nM) during pretreatment of Caco-2 cells ($P<0.01$). Similarly, inhibition of Cox-2 by 10 μM NS-398 significantly ($P<0.05$) decreased BBS-induced cell invasion by 67% on average (Figure 7c). BBS was also able to induce migration in Transwell assays of another human carcinoma cell line as HT29 to the same extent to that promoted by pre-incubation of those cells with PGE_2 (Figure 7d).

Discussion

Aberrant expression of Cox-2, BBS/GRP and GRP-R has been observed in a variety of tumors, including colorectal carcinomas (Preston *et al*., 1995; Carroll *et al*., 1999, 2000; Gupta and Dubois, 2001; Jensen *et al*., 2001) and several lines of evidence suggest a link among these proteins in cancer progression (Hecht *et al*., 1997; Guo *et al*., 2001; Iishi *et al*., 2003). We have analysed the potential association between BBS-mediated signaling and Cox-2 in colon carcinoma cells, demonstrating that BBS stimulation of Caco-2 cells leads to a rapid upregulation of Cox-2 expression at both mRNA and protein levels resulting in increased production of PGE_2. This effect can be attributed to BBS binding to a functional GRP-R that mediates mobilization of intracellular Ca^{2+}, likely through a $G\alpha_q$ protein, as it was blocked by the specific antagonist RC-3940-II. This effect was clearly observed at 100 nM BBS, which is higher than the reported IC_{50} for the binding to the receptor (around 1 nM) (Ryan *et al*., 1999). However, the affinity of labeled peptides for the receptor does not always correlate with the concentrations required to observe a functional effect. Moreover, peptidases present in the serum may interfere with BBS/GRP and GRP-R binding or function (Jensen *et al*., 2001), which may explain the discrepancies between data of binding affinities (measured in the absence of serum and after short periods of incubation) and concentrations reported for biological effects (requiring a longer period of incubation in the presence of serum). In this regard, similar concentrations of GRP/BBS as those used in our study have been reported for the induction of Cox-2 in other cell types (Hecht *et al*., 1997; Guo *et al*., 2001) as well as for regulating motility of Caco-2 cells upon BBS treatment (Glover *et al*., 2005).

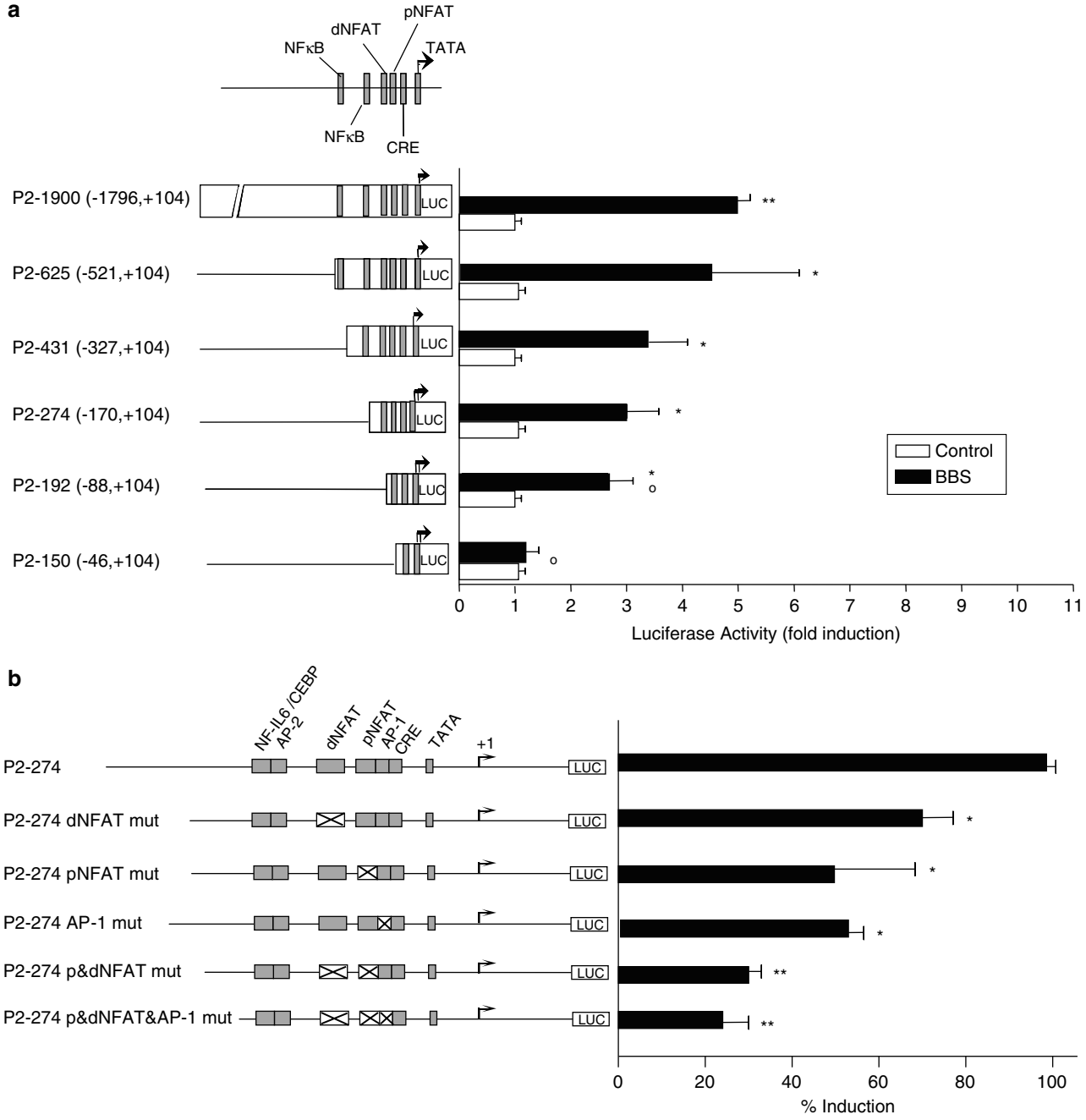

Figure 5 Analysis of Cox-2 transcriptional activity in Caco-2 cells stimulated with BBS. (**a**) Activation of Cox-2 gene promoter by BBS. Caco-2 cells were transfected with the different Cox-2 promoter reporter and cultured in the absence (Control) or presence of BBS (100 nM) for an additional 16-h period and assayed for luciferase activity. Results from triplicate assays of three independent experiments are shown as mean fold induction ± s.e. Deletions ranging from −1796 to −46 relative to the transcription start site of the Cox-2 promoter were used. Consensus sequences are denoted by boxes. The asterisks indicate statistically significant differences (*$P<0.05$; **$P<0.001$) between BBS and control samples; ($^{\circ}P<0.05$ respect to the longest construct P2-1900). (**b**) Role of NFAT in Cox-2 promoter induction by BBS. Caco-2 cells were transiently transfected with the P2-274-Cox-2 promoter construct, and the same construct containing dNFAT and/or pNFAT sites, and/or AP-1 site mutated (indicated by X). Three hours after transfection, the cells were stimulated with BBS (100 nM) for 16 h and luciferase activity was determined. The activity is expressed as percentage of induction relative to that achieved in cells transfected with the P2-274-Cox-2 promoter construct. One out of three separate experiments performed is shown.

Moreover, our results point out to the Ca^{2+}/Cn/NFAT pathway as an important signaling cascade in BBS-GRP-R-mediated events as Cox-2 induction. Previous reports have shown that NFAT proteins are key factors for the transcriptional regulation of the Cox-2 gene in many different cell types (Iniguez *et al*., 2000; Robida *et al*., 2000; Hernandez *et al*., 2001; Lara-Pezzi *et al*., 2002; Duque *et al*., 2005; Yiu and Toker, 2006).

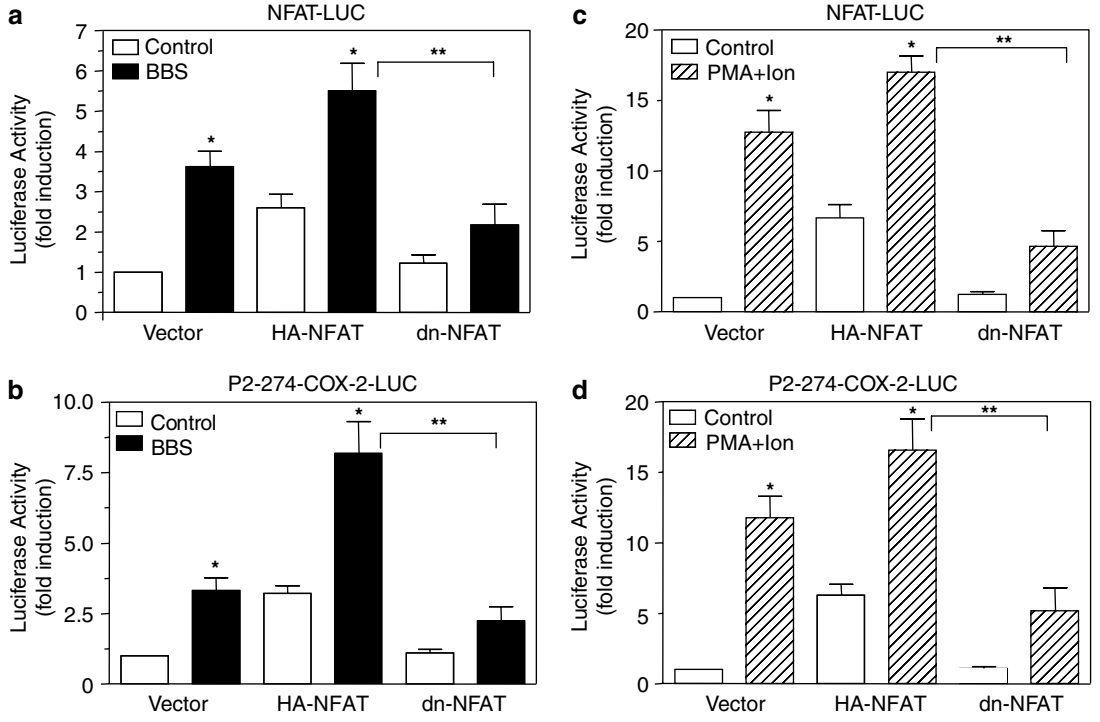

Figure 6 Involvement of the NFAT in Cox-2 induction by BBS in Caco-2 cells. Cells were transiently co-transfected with the P2-274-Cox-2-LUC or the NFAT-LUC reporter plasmid along with empty vector, HA-NFAT or dnNFAT. Transfected cells were untreated (Control), or treated with BBS (100 nM) or PMA (15 ng/ml) plus Ion (1 μM) for 16 h. Luciferase activity is expressed as fold induction (mean $\pm$ s.e. of triplicate determinations). Data are representative of three independent experiments. The asterisks indicate statistically significant differences ($P<0.05$; *between BBS (or PMA + Ion) and control samples; **between HA-NFAT and dnNFAT transfected expression vectors to BBS (or PMA + Ion)-treated cells).

Two NFAT binding sites in the Cox-2 promoter are essential for BBS-induced expression of Cox-2, one of them resembling an NFAT/AP-1 composite site. Mutation of either the NFAT or the AP-1 component of this site strongly diminished BBS-induced Cox-2 transcription in agreement with the well-known cooperation between NFAT and AP-1 necessary to drive efficiently transcription of a variety of NFAT-dependent genes (Hogan *et al*., 2003). In RIE/GPRP-R cells, BBS-mediated transcriptional induction of the Cox-2 gene is also calcium dependent, requiring activation of components of the AP-1 transcription factor complex, such as Fos/Jun proteins (Guo *et al*., 2001). Moreover, involvement of Gαq-coupled receptors as the GRP-R in the activation of NFAT and in the induction of NFAT-dependent genes has been described in a variety of cell types (Boss *et al*., 1996; Horsley and Pavlath, 2002; Liu *et al*., 2004). Noticeably, recent reports have proposed that NFAT proteins may play an important role in cancer, having shown a direct involvement of NFAT-mediated transcriptional regulation of different genes, including Cox-2 (Jauliac *et al*., 2002; Lara-Pezzi *et al*., 2002; Saurin *et al*., 2002; Neal and Clipstone, 2003; Holzmann *et al*., 2004; Yiu and Toker, 2006).

As mentioned above, GRP-R and Cox-2 expression in human colon cancer cells correlates with more invasive characteristics. Our findings show that in spite of the poor aggressiveness displayed by Caco-2 cells, their capacity to migrate towards Matrigel was greatly facilitated when they were pre-incubated with BBS. This enhanced invasion was induced through GRP-R, as the number of invading cells under BBS drastically decreased by treatment with the specific antagonist RC-3940-II. Furthermore, the Cox-2 selective inhibitor NS-398 was also able to reduce strongly BBS-promoted cell invasion, suggesting that this enzyme is involved in Caco-2 cells invasiveness. A similar effect of BBS on migration was observed with HT-29 colon carcinoma cells. BBS treatment may enhance cell invasion/migration by different mechanisms, some of which could be mediated through its ability to induce NFAT-dependent transcriptional activation of Cox-2. Previous reports have shown that BBS acts as an invasion-promoting agent in prostate and colon cancer cell lines, although the precise molecular mechanism(s) by which BBS causes these effects are unknown. BBS was shown to stimulate tyrosine phosphorylation of several focal adhesion proteins which mediate colon cancer cell motility (Aprikian *et al*., 1997; Rozengurt *et al*., 2002; Glover *et al*., 2005). BBS also enhances invasion of Isreco1 colon carcinoma cells through unidentified effector proteins downstream the Rho signaling pathway (Saurin *et al*., 2002). Moreover, BBS has been proved to induce the expression and activation of the extracellular matrix proteinases urokinase-type plasminogen activator and metalloproteinase-9, as well as the proangiogenic factors interleukin-8 and vascular endothelial growth factor, linked to increased metastatic

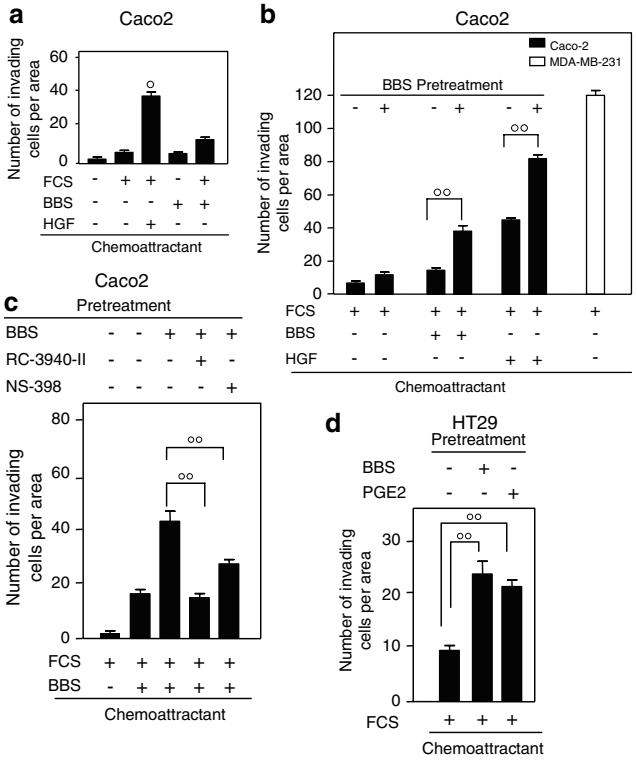

Figure 7 Effect of BBS on invasiveness of carcinoma cell lines. (**a**) Caco-2 cells (10^5) were serum-starved for 72 h and seeded in the upper compartment, whereas in the lower chamber there was DMEM supplemented with chemoattractants: 20% FCS with or without 100 ng/ml HGF and 100 nM BBS where indicated. (**b**) Caco-2 cells were serum-starved as above but in the presence or absence of 100 nM BBS as indicated. In the lower chamber, there was DMEM supplemented with 20% FCS plus, 100 ng/ml HGF or 100 nM BBS, where indicated. The MDA-MB-231 cell line was used as positive control in the invasion assay. (**c**) Caco-2 cells were serum-starved as above in the presence or absence of 100 nM BBS, with or without the addition of 10 nM RC-3940-II or 10 μM NS-398. In the lower chamber, there was DMEM supplemented with 20% FCS and 100 nM BBS, where indicated. (**d**) HT-29 cell were serum-starved and pre-incubated with 100 nM BBS or 1 μM PGE_2. In the lower chamber, there was DMEM supplemented with 20% FCS. After 3 days of culture, cells that passed across the membrane were fixed, stained and counted. Data represent the number (mean $\pm$ s.e. of four independent experiments, each performed in duplicate) of invading or migrating cells. Area corresponds to the surface occupied by the ocular grid at $\times 400$ magnification. $^{\circ}P<0.05$ and $^{\circ\circ}P<0.01$.

potential in prostate cancer cells (Festuccia *et al*., 1998, 2002; Levine *et al*., 2003). In addition, our results show the involvement of NFAT-mediated induction of genes implicated in tumor progression such as Cox-2 in the pro-tumoral effects of BBS. NFAT has been involved in promoting breast and colon carcinoma motility and invasion through Matrigel, mediating integrin-dependent events (Jauliac *et al*., 2002; Chen and O'Connor, 2005). In a recent report, Yiu and Toker (2006) have shown in breast cancer cells that increased expression and activity of NFAT-induced Cox-2 expression and PGE_2 production, resulting in increased invasion that was reduced by inhibiting Cox-2 expression or activity (Yiu and Toker, 2006). Multiple evidences support the involvement of Cox-2-derived PGs in tumor progression by their ability to enhance migration and invasion of carcinoma cells. Cox-2 expression in colon or breast cancer cell lines increases the expression of enzymes capable of digesting the basal membrane presumably contributing to the observed increased ability to invade through a layer of Matrigel (Tsujii *et al*., 1997). These effects are related to PG-mediated upregulation of metalloproteinases, β-catenin and diverse growth factors receptors (Tsujii *et al*., 1997; Pai *et al*., 2003; Singh *et al*., 2005). Nevertheless, it remains to be elucidated what is the contribution of NFAT/Cox-2 to the previously described events occurring upon BBS treatment in carcinoma cells. In agreement to our data showing inhibition of BBS-mediated enhanced invasion of Caco-2 cells by NS398, Iishi *et al*. (2003) reported that Nimesulide, a Cox-2 inhibitor, was able to suppress BBS-enhanced MMP-9 activity and peritoneal metastasis of intestinal adenocarcinomas in rats.

Based on our findings, we postulate that BBS-mediated cell growth, invasiveness and tumor progression in human colon cancer depend on the activation of the Ca^{2+}/Cn/NFAT signaling pathway that leads to an upregulation of genes involved in the tumoral phenotype such as Cox-2. Identifying changes in the gene expression pattern upon BBS treatment of colon carcinoma cells may further contribute to our understanding of the pathogenesis of colorectal carcinoma.

Materials and methods

Reagents

BBS, PMA and A23187 calcium Ion were purchased from Sigma. The Random Primers DNA Labelling System kit, Optiminimum essential medium (MEM) and Lipofectamine were obtained from Invitrogen (Carlsbad, CA, USA). enhanced chemiluminescence system was purchased from Amersham Pharmacia (Buckinghamshire, England). BCA Protein Assay reagent and Restore Western Blot Stripping buffer were obtained from Pierce (Rockford, IL, USA). The Luciferase Assay System and lysis buffer were purchased from Promega (Madison, WI, USA). Anti-Cox-2 antibody and the Cox-2 inhibitor NS398 were obtained from Alexis (San Diego, CA, USA). Anti-human β-actin and horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA), whereas anti-NFATc1 antibody was described previously (Iniguez *et al*., 2000). PGE_2 and the enzyme-linked immunosorbent assay (ELISA) kit for the determination of PGE_2 were obtained from Cayman. Fura-2/AM was purchased from Molecular Probes (Carlsbad, CA, USA). Trizol was obtained from Invitrogen. Nylon membranes were purchased from Schleicher and Schuell (Brentford, UK). CsA was obtained from Sandoz (Holzkirchen, Germany). Specific BBS antagonist (RC-3940-II) was kindly provided by Andrew Schally (Tulane University, New Orleans).

Cell culture

The Caco-2 human intestinal cell line was obtained from the American Type Culture Collection. Cells were cultured using MEM supplemented with 20% FCS for Caco-2 or 10% FCS for HT29, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM

sodium pyruvate, 2 mM L-glutamine and 1% non-essential amino acids at 37°C in a 5% CO_2 atmosphere. Cells were used between passages 15 and 45.

Intracellular calcium measurements
Agonist-induced changes in $[Ca^{2+}]_i$ were detected using the Ca^{2+}-sensitive dye Fura-2/AM as described by Hellmich *et al.* (1999). Briefly, cell monolayers at 80% confluence were trypsinized, washed and then loaded with 1 μM Fura-2/AM under continuous stirring for 30 min at 37°C. The cells (2×10^6/ml) were treated with 100 nM BBS or 10 nM RC-3940-II, and placed in an Aminco Bowman Series 2 spectrofluorometer (Thermo). The fluorescence signal of Fura-2 was recorded, with excitation and emission at 340 and 510 nm, respectively.

RNA isolation and Northern blot analysis
Total RNA was extracted from Caco-2 cells by using Trizol reagent according to the manufacturer's instructions. RNA samples (20 μg/lane) were separated on formaldehyde-agarose gels and blotted onto nylon membranes. The blots were hybridized with Cox-2 DNA probe labeled with [α-^{32}P]dCTP by random primer extension. After overnight hybridization at 65°C and thorough washing, the blots were exposed to X-ray film for autoradiography as described (Hernandez *et al.*, 2001). To ensure RNA integrity and to confirm equal loading among lanes, the filters were stripped and rehybridized with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Densitometric analysis was performed using NIH Image 1.62 software to quantify the results.

PGE_2 measurements
Cells were maintained for 12 h in culture medium supplemented with 0.5% FCS, then pretreated or not with 200 ng/ml CsA or 1 μM NS398 for 2 h, and further stimulated with 100 nM BBS for the indicated times. Media supernatants were collected and analysed for PGE_2 by ELISA (Hernandez *et al.*, 2001).

Immunoblot analysis
Immunoblotting was carried out as described elsewhere (Iniguez *et al.*, 2000). Cells were disrupted and solubilized extracts (20 μg) were separated in 6% (only for analysis of NFAT translocation to the nucleus) or 10% sodium dodecyl sulfate–polyacrylamide gels, and transferred to nitrocellulose filters. After blocking for 2 h with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20, the membranes were probed 2 h at 37°C with murine monoclonal antibody against Cox-2 (diluted 1:1000 in blocking buffer), polyclonal goat anti-human β actin antibody (1:1000), or polyclonal goat anti-human NFATc1 antibody (1:2000). The filters were washed and incubated with the corresponding secondary antibody linked to horseradish peroxidase at 1:10,000 dilution, and the stained bands were visualized by a chemiluminescent peroxide substrate.

Plasmid constructs
Human Cox-2 promoter constructs spanning from −1796 (P2-1900-LUC), −521 (P2-625-LUC), −327 (P2-431-LUC), −170 (P2-274-LUC), −88 (P2-192-LUC) and −46 (P2-150-LUC) to +104 bp relative to the transcription start site of the human Cox-2 gene and the P2-274-LUC plasmid with binding sites for NFAT, or AP-1, or both mutated were described (Iniguez *et al.*, 2000). The reporter construct NFAT-LUC contains three tandem copies of the NFAT binding site fused to the IL-2 minimal promoter. The EF-BOS-NFAT1 plasmid contains the cDNA encoding murine NFATc2, modified to encode an influenza HA peptide at the N-terminus of the expressed protein (Luo *et al.*, 1996). The pSH102CΔ418 expression vector (a kind gift from Gerald Crabtree, Stanford University, Stanford, CT, USA) derives from pBJ5 and encodes an NFATc1 deletion mutant (1–418) that functions as a dominant negative for all NFAT isoforms (Northrop *et al.*, 1994).

Transfection and luciferase assays
Caco-2 cells were transfected by Lipofectamine as described (Iniguez *et al.*, 2000). Briefly, exponential growing cells (1.25×10^5/well) cultured in 24-well plates were incubated for 3 h at 37°C with a mixture of 0.5–1 μg of the corresponding reporter plasmid and Lipofectamine-containing Opti-MEM. In co-transfection experiments, 0.15–1.5 μg of the corresponding expression plasmid was included. The total amount of DNA in each transfection was kept constant by using the empty expression vectors. Complete medium was then added to cells and incubated at 37°C for additional 16 h. Transfected cells were exposed to different stimuli as indicated. Then, cells were harvested and lysed. Luciferase activity was determined by using a luciferase assay system with a luminometer Monolight 2010 (Analytical Luminescence Laboratory). Transfection experiments were performed in triplicate. Data of luciferase activity are presented as fold induction (observed experimental relative luciferase units (RLU)/basal RLU in absence of any stimulus). Results were normalized for extract protein concentrations measured with a Bradford assay kit.

Electrophoretic mobility shift assay
Nuclear extracts were prepared from Caco-2 cells as described (Martinez-Martinez *et al.*, 1997) with minor modifications. In brief, 5 μg of nuclear protein was incubated with 1 μg of poly(dI–dC) DNA carrier in DNA binding buffer (10% (wt/vol) polyvinylethanol, 12.5% (vol/vol) glycerol, 50 mM Tris, pH 8, 2.5 mM dithiothreitol, 2.5 mM ethylenediaminetetraacetic acid) for 30 min at 4°C. Then, 10^5 counts per minute (c.p.m.) (10^8 c.p.m./μg) of the ^{32}P-labeled double-stranded oligonucleotide (2 μg) were added, and the reaction was incubated at room temperature for 30 min. For competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added before the addition of the probe. Supershift assays were performed by incubating nuclear extracts with either preimmune serum or anti-NFATc1 antibody for 15 min at 4°C before the addition of the probe. DNA–protein complexes were resolved by electrophoresis in 4% non-denaturing polyacrylamide gels and were subjected to autoradiography.

The following synthetic oligonucleotides (5′–3′) were used as probes/competitors in EMSAs (factor binding sites are underlined): tcgaCAAGGGGAGAGGAGGGAAAAATTTGTGGC (nucleotides −117 to −91 containing the putative dNFAT site of the human Cox-2 promoter); and tcgaCAAAAGGCGGAAAGAACAGTCATTTC (nucleotides −82 to −58 containing the putative AP-1-pNFAT site of the human Cox-2 promoter).

In vitro *cell migration and invasion assays*
Invasion and migration assays were carried out in Transwell filter chambers coated or not with Matrigel (Corning-Costar, Cambridge, MA, USA) (Albini *et al.*, 1987). Caco-2 cells were preincubated in FCS-free DMEM with 0.1% bovine serum albumin, in the presence or absence of 100 nM BBS, with or without the addition of BBS-R antagonist RC-3940-II (10 nM) or Cox-2 inhibitor NS-398 (10 μM), and then seeded (1×10^5) in the upper chamber equipped with a 12 μm Matrigel-coated

polycarbonate membrane. The culture medium was changed daily along 3 days. DMEM supplemented with 20% FCS was introduced into the lower compartment with or without recombinant human HGF (R&D Systems, Minneapolis, MN, USA) at 100 ng/ml, and with or without 100 nM BBS. To analyse the mechanisms associated with cell invasiveness, we used chemoattractants diluted in FCS-supplemented medium, as described by Kermorgant *et al*. (2001). In the lower chamber there was DMEM supplemented with 20% FCS. After 72 h of culture, the non-migratory cells on the upper surface of the filter were mechanically removed by scraping and the membranes fixed and stained with Diff-Quick (Baxter Scientific Products, McGraw Park, IL, USA). Cell migration was microscopically quantified by counting cells on the bottom surface of the filter at $\times 400$ magnification, using a calibrated ocular grid, in 16 representative areas per well. Experiments were carried out in duplicate and several independent sets of experiments for each type of migration assay were performed.

In the case of HT-29 only migration assays were carried out as above, but in absence of matrigel in a 8 μm pore size Polycarbonate Membrane Transwell Inserts (Corning-Costar, Cambridge, MA, USA). HT-29 cells were serum-starved and pre-incubated with 100 nM BBS or 1 μM PGE_2. Cell migration was microscopically quantified by counting cells on the bottom surface of the filter, analysing captured images at $\times 10$ magnification with the ImageJ v1.36b Software (Wayne Rasband, NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed by using GraphPad InStat 3.05 software. Comparisons between groups were made by using the one-way analysis of variance test. A difference between groups of $P<0.05$ was considered significant.

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