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Stromal COX-2 signaling activated by deoxycholic acid mediates proliferation and invasiveness of colorectal epithelial cancer cells

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ABSTRACT

COX-2 is a major regulator implicated in colonic cancer. However, how COX-2 signaling affects colonic carcinogenesis at cellular level is not clear. In this article, we investigated whether activation of COX-2 signaling by deoxycholic acid (DCA) in primary human normal and cancer associated fibroblasts play a significant role in regulation of proliferation and invasiveness of colonic epithelial cancer cells. Our results demonstrated while COX-2 signaling can be activated by DCA in both normal and cancer associated fibroblasts, the level of activation of COX-2 signaling is significantly greater in cancer associated fibroblasts than that in normal fibroblasts. In addition, we discovered that the proliferative and invasive potential of colonic epithelial cancer cells were much greater when the cells were co-cultured with cancer associated fibroblasts pre-treated with DCA than with normal fibroblasts pre-treated with DCA. Moreover, COX-2 siRNA attenuated the proliferative and invasive effect of both normal and cancer associate fibroblasts pre-treated with DCA on the colonic cancer cells. Further studies indicated that the activation of COX-2 signaling by DCA is through protein kinase C signaling. We speculate that activation of COX-2 signaling especially in cancer associated fibroblasts promotes progression of colonic cancer.

1. Introduction

Prostaglandins are small lipid messengers, which participate in pain, inflammation, and colorectal carcinogenesis [1]. The main form of prostaglandins is prostaglandin E_2 (PGE_2). Synthesis of prostaglandins is catalyzed by the enzyme cyclooxygenase (COX), including COX-1 and COX-2. COX-1 is constitutively expressed in all tissues and is involved in physiological functions [2], while COX-2 is a rate-limiting enzyme for generation of prostaglandins [3–7]. Elevated production of PGE_2 is linked to malignant transformation and progression in ovarian and pancreatic cancer [8,9]. However, the origin of COX-2 and its product, PGE_2, is still controversial.

Traditionally, the causation of colonic cancers were attributed largely to genetic alterations in the epithelial compartment [10]. However, recent studies demonstrated that stromal fibroblasts are actively involved in regulation of COX-2 signaling [11–15], and in occurrence of cancer [16–19].

The stroma is the compartment that serves as the connective tissue framework, in which the majority of the cells are fibroblasts. A recent study revealed that cancer-associated fibroblasts (CAFs) promoted the initiation and development of epithelial ovarian cancer [19]. We have shown that colonic fibroblasts are a potent source of inducible COX-2 expression and PGE_2 synthesis and that inflammatory factors such as IL1-β and TNF-α stimulated COX-2 expression and PGE_2 production in colonic fibroblasts [12–14]. Maximal levels of COX-2 expression and PGE_2 production were far greater in CAFs initiated from biopsies of colonic cancers than in normal fibroblasts (NFs) initiated from normal colorectums [13]. However, the luminal factors such as bile acids that regulate stromal fibroblast production of paracrine factors are poorly understood.

The primary bile acids, cholic and chenodeoxycholic acid, are synthesized in hepatocytes from cholesterol and conjugated with glycine or taurine before their secretion in the bile. After bacterial deconjugation in the distal small intestine, most bile acids are absorbed and returned to the liver in the enterohepatic circulation. Unabsorbed bile acids are dehydroxylated by colonic bacteria to become secondary dihydroxy bile acids, of which deoxycholic acid (DCA) is the predominant fecal bile acid. DCA was shown to be transcriptional activators of COX-2 in an esophageal cancer cell line [20] and in the human colon cancer cell line HCT-116 [21].

Abbreviations: DCA, deoxycholic acid; COX, cyclooxygenase; PGE_2, prostaglandin E_2; PKC, protein kinase C; STA, staurosporine; BIM, bisindoylmalemide I.

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Previously, we reported that DCA induced COX-2 expression and its product PGE_2 synthesis in colonic fibroblasts [13]. But it is unclear whether activation of COX-2 signaling in the fibroblasts affects behavior of colonic epithelial cancer, including proliferation and invasiveness.

The aim of this study is to investigate whether activation of COX-2 signaling by DCA in NFs and CAFs modulates the behavior of colonic epithelial cancer cells.

2. Materials and methods

2.1. Materials

Human colonic cancer cell lines (HT29, Caco2, HCA7 and HCT116) were obtained from American Type Culture Collection (Manassas, VA, USA). Cell culture reagents and DCA were purchased from Sigma (St. Louis, MO, USA). COX-2 antibody was obtained from Cayman Chemical (Cayman, Ann Arbor, MI, USA). Real-time PCR primer-probes were purchased from Applied Biosystems (Foster City, CA, USA). PGE_2 radioimmunoassay kits were obtained from Amersham (Arlington Heights, IL, USA). Control scRNA, COX-2 siRNA and HiPerFect® siRNA transfection reagents were obtained from Qiagen (Valencia, CA, USA). Primary fibroblast cultures were initiated from pinch biopsies obtained, with approval of the Institutional Review Board of the Buffalo Veterans Affairs Medical Center, USA, at colonoscopies performed in the course of routine clinical care.

2.2. siRNA transfection

For the short pulse siRNA knockdown, parallel cultures were subjected to scRNA or COX-2 siRNA transfection following our published protocol [22]. The transfected cells were cultured for 24 h before treatment of PBS or DCA (300 μM) for 24 h.

2.3. Real-time PCR analysis of COX-2 mRNA

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was reverse-transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA of each cell sample was amplified by real-time RT-PCR using specific primer-probe mixtures and DNA polymerase in 7000 Real-time PCR System (Applied Biosystems) following our published protocol [22].

2.4. Western blotting analysis of COX-2 protein

Western blotting analysis was performed as described previously [22]. The resulting bands of COX-2 were analyzed densitometrically and the protein loading difference was corrected by the density of β-actin.

2.5. Immunostaining

Fibroblast cultures were air-dried and fixed in 4% formaldehyde, rehydrated in PBS, incubated with 0.2% Triton X-100 for 15 min. After incubation with 2% BSA to block non-specific staining for 30 min, they were incubated with COX-2 antibody (at 1:50 dilution) for 16 h at 4 °C. After washes, the samples were incubated with corresponding Alexa Fluor-conjugated secondary IgG for 60 min. The samples were then counterstained with Hoechst 33342 and analyzed with Zeiss LSM 700 confocal microscope (Thornhood, NY, USA).

2.6. PGE_2 assay

Fibroblasts were seeded in 24-well plastic culture plates at a density of 5×10^5 cells/cm^2 and were cultured for 1 week in DMEM supplemented with 10% FBS. It is well recognized that serum is a potent inducer of COX-2 and, thus, PGE_2. Therefore, 24 h before administering DCA or other treatments to fibroblast cultures, medium was replaced with DMEM supplemented with 1% FBS. To determine the effect of DCA, selected cultures were incubated in medium supplemented with DCA (300 μM). For the final 30 min before harvesting of cultures, medium was replaced with PBS supplemented with 1% gelatin. Test compounds were present during this final incubation period. PGE_2 levels in harvested PBS-gelatin were determined using radioimmunoassay kits (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions. Protein concentration in the harvest was determined by the method of Bradford.

2.7. Co-culture and proliferation assay

Fibroblasts (1×10^7/well, grew until 90% confluence) were first prepared in the lower compartment. The fibroblasts were pre-treated with PBS, DCA (300 μM) for 24 h. Colonic cancer cells (1×10^6/well) were later cultured in upper compartment of 12 mm Transwell chambers (0.4 μm, Corning Costar Co., Cambridge, MA, USA). 4 h prior to termination of the experiment, ^{3}[H]thymidine (0.8 μCi/apical Transwell compartment) was added. Cultures were determined from the incorporation of ^{3}[H]thymidine into cellular DNA. Cultures without fibroblasts were used as controls.

2.8. Co-culture and invasion assay

Colonic cancer cells (1×10^5/well) were grown on the filters of chambers with 8 μm pore size coated with Matrigel (Biocoat chambers, Becton Dickson, Bedford, MA, USA) for 24 h in upper compartment, with 90% confluent fibroblasts in the lower, pre-treated with PBS, DCA (300 μM) for 24 h. Cultures without fibroblasts were used as the controls. Cells on the upper surface of the filters were removed and cells adhering to the undersurface of the filters were counted.

2.9. PKC and PKC inhibitor assay

For PKC activity, we followed our previously published protocol [11–15]. In further experiments, a nonselective protein kinase inhibitor, staurosporine (STA, 20 μM), or a selective PKC inhibitor, bisindoylmalemide I (BIM, 10 μM), was combined with DCA. Their inhibitory effects on PGE_2 synthesis were examined as described above.

2.10. Cell viability

Cell viability was determined by trypan blue exclusion [12]. Cells were seeded at the same time from a single parent culture. Cultures were incubated without (control) or with DCA. Treated and control cultures were harvested at the same time, after careful washing to remove cells that had detached during incubation. Harvested cells were incubated with trypan blue and counted using a hemocytometer. From each culture, four fields of triplicate preparations were counted for the percentage of cells that excluded the dye. Cell viability was >95% after all treatment periods (data not shown).

2.11. Statistics

All summary data were reported as mean ± S.E. calculated for each group and compared using Analysis of Variance test and Student's unpaired *t*-test by MicroSoft Excel™ (MicroSoft, Redmont, WA, USA). Test results were reported as two-tailed *p* values, where $p < 0.05$ was considered statistically significant.

3. Results

3.1. COX-2 was detected in resting CAFs and both NFs and CAFs after stimulation of DCA, but not in resting NFs

Previously causation of colorectal cancers was attributed largely to alteration in the colonic epithelial cells. However, research evidence indicated that COX-2 was expressed in the stromal cells of intestinal microadenomas in genetically modified mice [17] and human colorectal adenomas [23]. Herein, we demonstrated that significant COX-2 expression was detected in resting CAFs but not in resting NFs, and in NFs and CAFs after induction by DCA (Fig. 1A).

3.2. DCA elicited significant higher expression of COX-2 mRNA and protein in CAFs than that in NFs

Resting CAFs expressed more (5-fold) COX-2 mRNA than resting NFs. Stimulation by DCA elicited apparent up-regulation of COX-2 mRNA in NFs (15-fold) but robust up-regulation of COX-2 mRNA in CAFs (30-fold when compared to the control of NFs, 6-fold when compared to the control of CAFs, Fig. 1B). Interestingly, COX-2 signaling was not activated in resting NFs, but was already activated in resting CAFs. The activation of COX-2 mRNA by DCA was greater in CAFs than that in NFs. In addition, resting CAFs expressed more (4-fold) COX-2 protein than resting NFs. Stimulation by DCA elicited significant up-regulation of COX-2 protein in NF (5-fold) but robust up-regulation of COX-2 protein CAFs (13-fold when compared to the control of NFs, 3-fold when compared to the control of CAFs, Fig. 1C).

3.3. DCA elicited corresponding higher production of PGE_2 in CAFs than that in NFs

Stimulation by DCA elicited significant up-regulation of PGE_2 synthesis in NF (8-fold) but robust up-regulation of PGE_2 synthesis in CAFs (34-fold when compared to that of NFs, 4-fold when compared to that of CAFs, Fig. 1D).

3.4. CAFs activated by DCA promoted greater proliferative and invasive responses of colonic epithelial cancer cells than NFs

CAFs, with or without treatment of DCA, elicited quantitatively greater proliferation responses (3- and 4-fold increase respectively) in human colonic epithelial cancer cells than those from NFs (1.5- and 2.3-fold increase respectively, Fig. 2A and B). Correspondingly, CAFs, with or without treatment of DCA, elicited quantitatively greater invasive responses (approximately 40 and 60 more invasive cells/view respectively than the controls) in human colonic epithelial cancer cells than those initiated from NFs (approximately 10 and 20 more invasive cells/view respectively than the controls) (Fig. 2C and D).

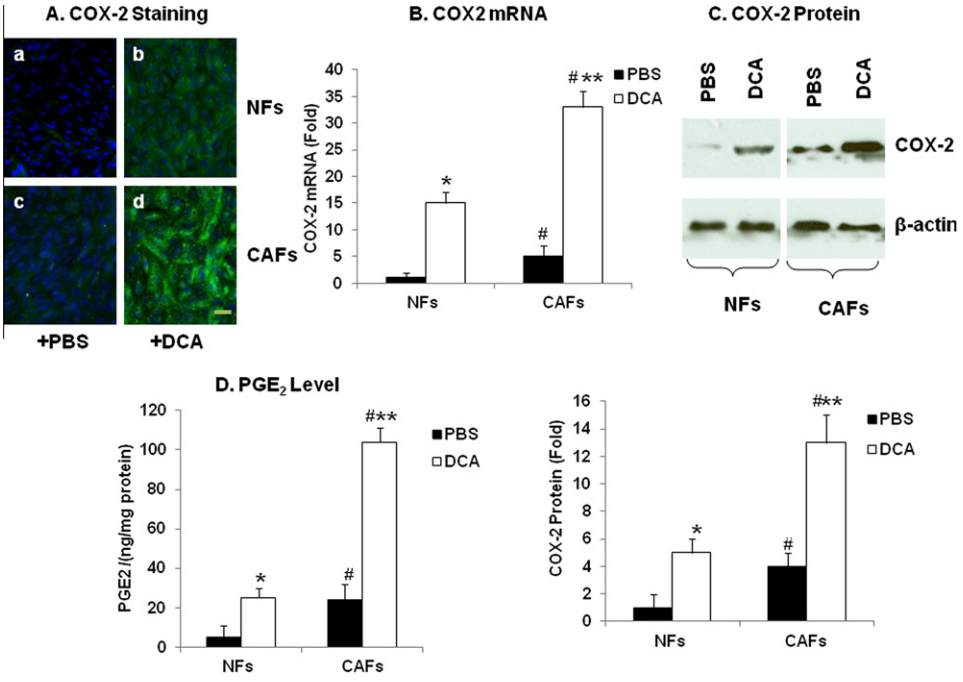

Fig. 1. DCA activates COX-2 signaling in NFs and CAFs. (A) Cytoplasmic expression of COX-2 in NFs and CAFs. Strong COX-2 expression was detected in NFs treated with DCA (A, b) and CAFs with or without DCA (A, c and d). (B) DCA stimulates the expression of COX-2 mRNA in NFs and CAFs. NFs or CAFs were cultured in medium with or without DCA (300 μM). Stimulation by DCA elicited apparent up-regulation of COX-2 mRNA in NFs, but robust up-regulation of COX-2 mRNA in CAFs ($n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ respectively). The level of COX-2 in CAFs were much higher than that in NFs ($n = 4$, $^{\#}p < 0.05$). (C) DCA promoted the expression of COX-2 protein in NFs and CAFs. NFs or CAFs were cultured in medium with or without DCA (300 μM). Stimulation by DCA elicited significant up-regulation of COX-2 protein in NFs, but robust up-regulation of COX-2 protein in CAFs ($n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ respectively). The level of COX-2 protein in CAFs was greater that in NFs ($n = 4$, $^{\#}p < 0.05$). (D) DCA promoted synthesis of PGE_2 in NFs and CAFs. NFs or CAFs were cultured in medium with or without DCA (300 μM). Stimulation by DCA elicited significant up-regulation of PGE_2 synthesis in NFs but profound up-regulation of synthesis in CAFs ($n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ respectively). The level of COX-2 protein in CAFs was greater than that in NFs ($n = 4$, $^{\#}p < 0.05$).

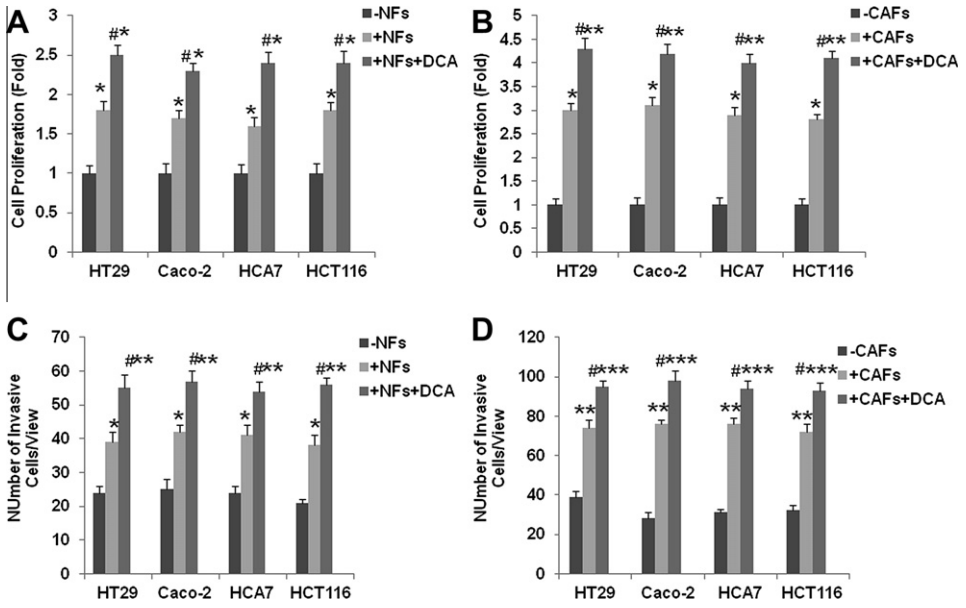

Fig. 2. The effect of NFs and CAFs pre-treated with DCA on proliferation and invasiveness of colonic epithelial cancer cells. (A and B) NFs and CAFs pre-treated with DCA promote proliferation of colonic epithelial cancer cells. Colonic cancer cell lines (HT29, etc.) and NFs or CAFs, respectively, were cultured in the upper and lower compartments of Snapwell co-culture chambers (0.4 μm pores). In selected experiments, some cultures were stimulated with DCA (300 μM). The proliferation of colonic epithelial cancer cell proliferation was measured by incorporation of ^{3}[H]thymidine. CAFs (B), with or without treatment of DCA, elicited quantitatively greater proliferation responses than NFs (A) ($n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared to the −NFs control; $^{\#}p < 0.05$ when compared to the +NFs group). (C and D) NFs and CAFs pre-treated with DCA promote invasiveness of colonic epithelial cancer cells. NFs (C) or CAFs (D) and epithelial cells were cultured in separate compartments of Matrigel chambers (Becton Dickinson, Bedford, MA, USA), separated by a filter with 0.8 μm pore size. The upper surface of the filter, on which epithelial cells were cultured, is coated with Matrigel. The lower compartment contained no cells (Control), or fibroblast cultures in unsupplemented medium or medium supplemented with DCA (300 μM). After coculture for 24 h, cells on the upper surface of filters were removed. Cells on the lower surface, which had invaded through the Matrigel layer and the filter pores, were counted ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ respectively when compared to the −NFs control; $^{\#}p < 0.05$ when compared to the +NFs control).

3.5. COX-2 siRNA blocked most of the proliferative and invasive effects of NFs and CAFs pre-treated with DCA on colonic epithelial cancer cells

Our results indicated that COX-2 siRNA blocked most of the paracrine effect of NFs and CAFs on proliferative responses in colonic epithelial cancer cells with or without stimulation of DCA (Fig. 3A and B). Interestingly, COX-2 siRNA also attenuated most of the paracrine effect of NFs and CAFs on invasive responses in colonic epithelial cancer cells with or without stimulation of DCA (Fig. 3C and D).

3.6. DCA-stimulated PGE_2 synthesis was mediated by protein kinase C (PKC), not Ca^{2+} signaling

The PKC superfamily participates in a wide array of cellular responses. Our results indicated that activation of PKC by DCA peaked at 5 min in both NFs and CAFs (Fig. 4A and B). Immediate addition of protein kinase inhibitor, STA (not shown) or PKC inhibitor BIM (20 and 10 μM respectively) to the cultures treated with DCA for 24 h completely blocked activation of PKC (not shown), and attenuated DCA-stimulated synthesis of PGE_2 in both NFs and CAFs (Fig. 4C and D). DCA did not induce any Ca^{2+} Signaling in NFs and CAFs (not shown).

4. Discussion

Previously causation of colorectal cancers was attributed largely to genetic alterations within the epithelial compartment [10]. However, research evidence indicated that COX-2 was expressed in the stromal but not epithelial cells of intestinal microadenomas in genetically modified mice [17] and human colorectal adenomas [23]. In this article, we demonstrated that significant COX-2 expression was detected in resting CAFs, but not in resting NFs, indicating that COX-2 signaling is already activated in resting CAFs, not in resting NFs (Fig. 1). In addition, DCA induced significant upregulation of COX-2 in NFs, but profound upregulation in CAFs, suggesting that stromal fibroblasts, especially CAFs, are origin of COX-2 (Fig. 1). Further analysis indicated that DCA elicited significant up-regulation of COX-2 mRNA in NFs, but robust up-regulation of COX-2 mRNA in CAFs, indicating that stromal fibroblasts, especially CAFs, are major sources of COX-2 (Fig. 1). Correspondingly, resting CAFs expressed profoundly greater COX-2 protein than resting NFs. Stimulation by DCA elicited significant up-regulation of COX-2 protein in NFs but profound up-regulation of COX-2 protein in CAFs (Fig. 1), confirming that CAFs are important sources of COX-2. Those results were similar to our previous reports that TNF-α and IL-1β induced COX-2 expression in various colonic fibroblasts [12–14]. Further analysis indicated that DCA also elicited corresponding higher production of PGE_2 in CAFs than that in NFs (Fig. 1), suggesting that stromal fibroblasts, especially CAFs, are the source of PGE_2.

To determine whether the fibroblasts play a critical role in carcinogenesis of colorectal cancer *in vitro*, we investigated whether NFs and CAFs stimulated by DCA promotes proliferative and invasive responses of human colonic epithelial cancer cells. Interestingly, CAFs pre-treated with DCA promoted quantitatively greater proliferative responses of colonic epithelial cancer cells than NFs (Fig. 2) indicating that paracrine factors secreted by CAFs promote growth of human colonic epithelial cancer cells. In addition, paracrines secreted by CAFs pre-treated with DCA promoted quantitatively greater invasive responses in human colonic epithelial cancer cells than paracrines secreted by NFs treated with DCA (Fig. 2), suggesting that paracrine factors secreted by fibroblasts also promote invasiveness of the colonic epithelial cancer cells. A previous report indicated that PGE_2 promoted malignant transformation and progression in ovarian cancer [8], thus we speculated the paracrine factors might include the major form of prostaglandins, PGE_2.

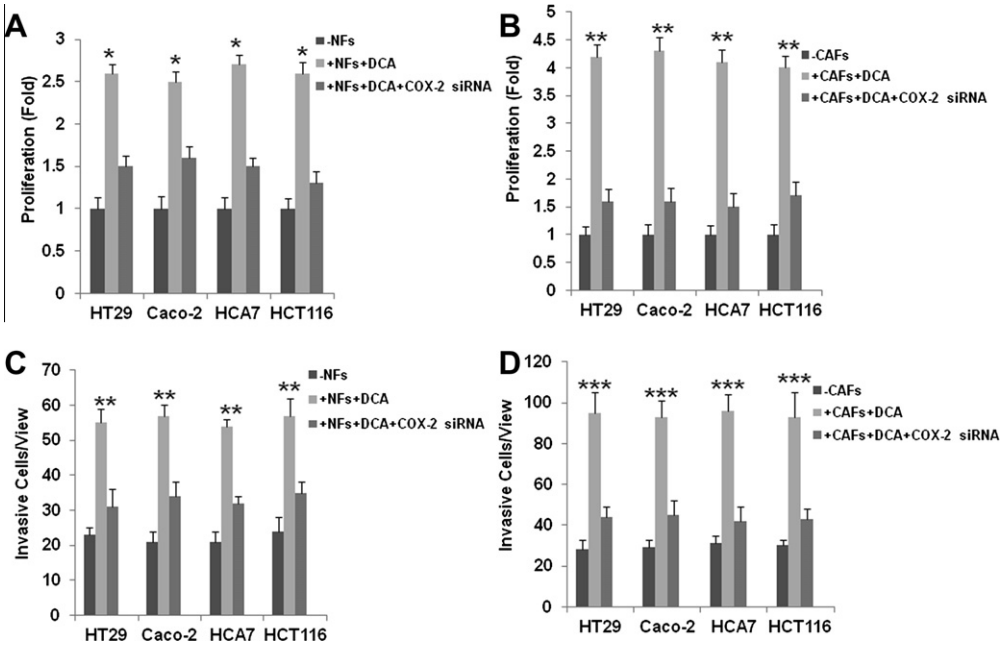

Fig. 3. COX-2 siRNA attenuated proliferation and invasiveness of colonic epithelial cancer cells induced by paracrines secreted by NFs and CAFs pretreated with DCA. (A and B) COX-2 siRNA blocked proliferation of colonic epithelial cancer cells induced by paracrines secreted by NFs and CAFs pre-treated with DCA. COX-2 siRNA was added at 24 h before treatments. COX-2 siRNA blocked most of the paracrine effect from NFs (A) and CAFs (B) on proliferation of colonic epithelial cancer cells with or without stimulation of DCA (300 μM, $n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ respectively). (C and D) COX-2 siRNA reduced invasiveness of colonic epithelial cancer cells induced by paracrines secreted by NFs and CAFs pre-treated with DCA. COX-2 siRNA was added 24 h before treatments. COX-2 siRNA also blocked most of the effect of NFs (C) and CAFs (D) on invasion of colonic epithelial cancer cells with or without stimulation of DCA (300 μM, $n = 4$, $^{**}p < 0.01$ and $^{***}p < 0.001$ respectively).

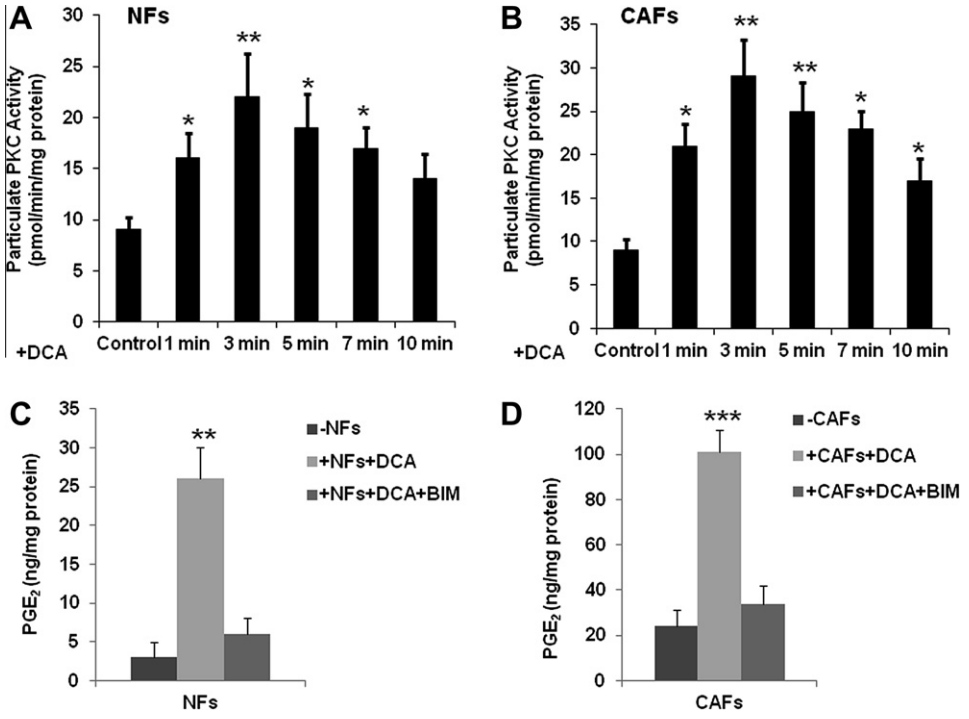

Fig. 4. PKC was activated by DCA in NFs and CAFs, and blockade of PKC activity by PKC inhibitor, BIM, inhibited production of PGE_2. (A and B) PKC was activated by DCA in NFs and CAFs. NFs (A) and CAFs (B) were exposed to DCA (300 μM) for periods of 1–10 min. Cell homogenates were pelleted, and particulate fractions were resolubilized. PKC activity of these samples was determined from transfer of [−32]ATP to a PKC-specific peptide and were expressed as pmol/min/mg protein. Bars represent the mean of results from 4 separated cultures ± SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared to the respective control. (C and D) Inhibition of PKC activity reduced production of PGE_2. NFs (C) and CAFs (D) were co-incubated with DCA 300 μM) and staurosporine (STA, 20 μM, not shown) or bisindoylmalemide (BIM, 10 μM) for 24 h. PGE_2 levels in harvested medium were determined by RIA. Bars represent the mean of results from 4 separated cultures ± SE. $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to the DCA- control.

To determine whether activation of COX-2 signaling was the major paracrine factor in NFs and CAFs promoting proliferation and invasiveness of colonic epithelial cancer cells, we used COX-2 siRNA to block COX-2 expression and PGE_2 production from NFs and CAFs. Our results indicated that the proliferative and invasive effect of NFs and CAFs pre-treated with DCA on colonic epithelial cancer cells was mostly attenuated by COX-2 siRNA (Fig. 3), suggesting that activation of COX-2 signaling in the fibroblasts is largely responsible for the proliferation and invasive responses in the colonic epithelial cancer cells. Those results were confirmed by treatment of COX-2 inhibitor, NS398, to the fibroblast cultures (not shown). Our conclusion is in line with a previous report that inhibition of COX-2 signaling significantly decreased proliferation of Barrett's esophageal epithelial cells by inhibition of PGE_2 production [24].

We recognize that fibroblasts could retain significant ability to induce epithelial cell proliferation by non-COX-2 related paracrine mechanisms. For example, transforming growth factor-β_2 and hepatocytes growth factor enhance epithelial cell proliferation and both can be synthesized by fibroblasts [25]. However, we believe that those factors might only play a minor role for the proliferative and invasive effect elicited by the colonic fibroblasts pre-treated with DCA since specific COX-2 siRNA inhibited most of proliferative and invasive effect elicited by both NFs and CAFs pre-treated with DCA.

To further study the mechanism, we found that activation of PKC by DCA peaked at 5 min in both NFs and CAFs (Fig. 4). Immediate addition of PKC inhibitor BIM to the cultures treated with DCA for 24 h blocked most of DCA stimulated synthesis of PGE_2 in both NFs and CAFs (Fig. 4), suggesting that DCA-stimulated COX-2 gene expression is PKC-dependent, similar to our reports that lipopolysaccharide and deoxycholate activated COX-2 gene expression is PKC-dependent [11–15].

In summary, the scientific relevance can be summarized as follows: CAFs are the potent source of COX-2 expression, which could be greatly induced by DCA; activation of stromal COX-2 signaling is largely responsible for change of proliferation and invasive potential of human colonic epithelial cancer cells *in vitro*.

Acknowledgments

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