COX2 Inhibitor NS398 Reduces HT-29 Cell Invasiveness by Modulating Signaling Pathways Mediated by EGFR and HIF1-α

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Abstract. *Background: Signals from the tumor micro-environment (hypoxia, growth factors) are known to induce an invasive phenotype. Cyclooxygenase-2 (COX2) overexpression, involved in colorectal carcinoma (CRC) progression, is also associated with epidermal growth factor receptor (EGFR) up-regulation. The present study investigated whether inhibition of COX2 may affect, under normoxia and hypoxia, EGF-induced cell proliferation and invasiveness by using immunoblotting, trypan blue assay, Boyden chamber assay and zymography. Results: The proliferative and invasive activity of HT-29 cells was enhanced under hypoxia. COX2 expression was increased after epidermal growth factor (EGF) stimulation under both hypoxia and normoxia, expression that was efficiently reduced by the COX2 inhibitor NS398. Under normoxia, NS398 reduced signalling pathways induced by EGF [phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT), extracellular-signal-regulated kinases (ERKs)], while under hypoxia, EGF stimulation and NS398 treatment was associated with HIF-1α expression. Under both conditions, NS398 was able to inhibit cell invasiveness and matrix-metalloproteinase-2 release. Conclusion: COX2 inhibition can contribute to reducing cell aggressiveness through interfering with EGF- and hypoxia-mediated signaling.*

Abbreviations: Colorectal carcinoma (CRC), cyclooxygenase-2 (COX2), epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), epithelial-to-mesenchymal transition (EMT), hypoxia-inducible factors (HIFs), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT), matrix metalloproteinases (MMPs).

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Key Words: HT-29, COX2, EGFR, hypoxia, invasiveness.

Colorectal carcinoma (CRC) is one of the major causes of cancer mortality worldwide (1), showing poor outcome essentially due to liver metastatic disease. Progression of CRC is based on a well-defined series of steps promoting increased motility and invasiveness of tumour cells, but can also be considered the result of a complex cross-talk between epithelial and mesenchymal cells (2). In this respect, different cell types in the microenvironment can play a pivotal role in cancer progression, through the interaction with tumor epithelial cells, either physically or *via* the secretion of paracrine signaling molecules. Along these lines, a major route of communication between these cells may occur through de-regulation of growth factor availability, including epidermal growth factor. Hypoxia causes up-regulation of epidermal growth factor receptor (EGFR) expression (3), and increased expression of EGFR highly correlates with a more severe histological grading, poor prognosis and resistance to therapy in patients with colon cancer and is believed to be sufficient to promote metastasis in animal models (4). Since EGFR activity highly correlates with tumour progression, EGFR-targeted therapy has been reported to prevent stimulation of the receptor by endogenous ligands, and rely on the inhibition of EGFR phosphorylation and de-regulation of its effector pathways, including the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) often involved in tumorigenesis (5-7).

Hypoxic areas are common and usually heterogeneously distributed within the neoplastic mass. Hypoxia is currently proposed as an additional putative environmental regulator able to promote tumor aggressiveness. Cellular response to hypoxia is primarily mediated by a family of hetero-dimeric hypoxia-inducible factors (HIFs), composed of a constitutively expressed β-subunit (HIF-β) and by oxygen-sensitive α-subunits (HIF-1α, HIF-2α and HIF-3α), which under hypoxic conditions, heterodimerize with HIF-β and translocate to the nucleus. Under normoxia, HIF-1α is rapidly degraded, whereas adaptation to hypoxia occurs

through stabilization and transcriptional activation of several genes that allow cells to survive in an oxygen-deprived environment (8). α-Subunits have been proposed to exert distinct roles in tumorigenesis (9), and in particular HIF-1α plays a critical role in human colon cancer (10).

Development of malignant tumour is also closely associated with degradation of extracellular matrix (ECM) by extracellular proteinases, among which matrix metalloproteinases (MMPs). Besides their degrading ability, MMPs also cleave cell surface molecules and other peri-cellular non-matrix proteins, participating in numerous biological processes and regulating cell behaviour (11). Up-regulation of MMP2 and MMP9, in particular, has been shown to play a key role in colon cancer progression, both in animal models and in human patients (11), favouring infiltration of the stroma surrounding the initial neoplastic focus (12).

A crucial role during development of the majority of colorectal tumours is also played by the overexpression of cyclooxygenase-2 (COX2) (13), a key enzyme in prostaglandin E_2 (PGE_2) synthesis, also identified as a direct transcriptional target for HIF-1α in colorectal tumour cells (14, 15). De-regulation of the COX2/PGE_2 pathway in colon tumourigenesis has been reported to affect ERK/MAPK, PI3K/AKT (16-18) and EGFR (19) signaling pathways which, in turn, can promote expression of COX2, also known to be up-regulated by specific tumour microenvironmental signals, including EGF and hypoxia (15). Furthermore, both hypoxia and EGF have been demonstrated to induce epithelial-to-mesenchymal transition (EMT) (20, 21), a process leading cancer cells to become more invasive and aggressive.

On the basis of these premises, this study was designed in order to further investigate whether inhibition of COX2 may differently affect, under normoxic and hypoxic conditions, EGF-induced cell proliferation and invasiveness of human colon cancer cells *in vitro*.

Materials and Methods

Materials. EGF and mouse monoclonal antibody for β-actin was purchased from Sigma Chemical Co. (MO, USA). Rabbit polyclonal antibodies for p-AKT, p-ERK1/2, N-cadherin, E-cadherin, HIF-1α, and EGFR, mouse monoclonal antibodies for PI3Kp85α, β-catenin, phosphatase and tensin homolog (PTEN) and COX2, goat polyclonal antibodies for p-EGFR, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS398) was from Sigma Chemicals Co., solubilized in dimethyl sulfoxide (DMSO) and used at a final concentration of DMSO that never exceeded 0.1%. The enhanced chemiluminescence reagent and nitrocellulose membrane (Hybond-C extra) were from GE Healthcare Europe GmbH (Milano, Italy). Matrigel was provided by BD Biosciences Italia (Buccinasco, Milan, Italy). All other reagents were from Sigma Chemicals Co.

Cell culture. The HT-29 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B, at 37˚C in a humidified atmosphere of 95% air/5% CO2. In experiments designed to evaluate the role of hypoxia, cells were first seeded under normoxic conditions to obtain the desired sub-confluence level (65-70%) and then were incubated under strictly controlled hypoxic conditions (3% O_2). For treatments, cells were allowed to adhere to the substratum, incubated for 24 h with serum-free insulin-free (SFIF) medium and then exposed for different time periods to EGF (100 ng/ml) and NS398 (20 and 75 μM), alone or in combination, under normoxic and hypoxic conditions.

Cell viability and proliferation. Cells were seeded in 12-well culture plates and treated as required. Aliquots of cell suspension were incubated with trypan blue solution (0.5% in NaCl) for 5 min to assess cell viability. Finally, cells were transferred to a Bürker chamber and counted under light microscopy. Dead cells were defined as those stained with the dye. For cell proliferation, cells were harvested at each time point and then cells numbers were manually counted under a microscope.

Western blotting. Protein levels were measured using a commercially available assay (Protein Assay Kit 2, Bio-Rad Laboratories, Milano, Italy) with bovine serum albumin as a standard. Total and nuclear extracts were obtained as previously detailed (18) and then subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis on 12, 10 or 7.5% acrylamide gels (Bio-Rad Laboratories, Milano, Italy). The blots were incubated first with desired primary antibody and then with HRP-conjugated secondary antibodies in Tris-buffered saline-Tween containing 2% (wt/vol) non-fat dry milk, and developed with the enhanced chemiluminescence reagent. Band intensities were quantified by densitometry (VersaDoc Imaging System 3000; Bio-Rad Laboratories, Milano, Italy) and the expression of proteins is reported as a proportion of that for β-actin protein expression used to monitor gel loading.

Cell invasion assay. Cell invasion was analyzed by employing Boyden chambers equipped with polyvinyl-pyrrolidone-free polycarbonate filters (8 μm porosity) that were coated with 50 μg/ml of Matrigel solution. The filters were then fixed in ice-cold methanol and stained with crystal violet solution. Cell invasion was quantified by counting, under a Zeiss microscope (Oberkochen, Germany) equipped with bright-field optics (×400 final magnification), crystal violet-stained cells that invaded Matrigel. For each filter, cells in 10 randomly chosen fields were counted and expressed as the number of invading cells per high-power field.

Gelatin zymography. Analysis of the activity of MMP2 (72-kDa type IV collagenase) was performed on culture medium by means of gelatin zymography in 0.1% gelatin-30% acrylamide gels. Equal amounts of culture medium were loaded in each lane properly diluted in sample buffer 2× (0.5 M Tris HCl pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue, distilled water, without β-mercaptoethanol) and the overall procedure was performed as previously described (22). Gelatinolytic activity of MMP2 was detected as clear zones of lysis against a blue background. Protein

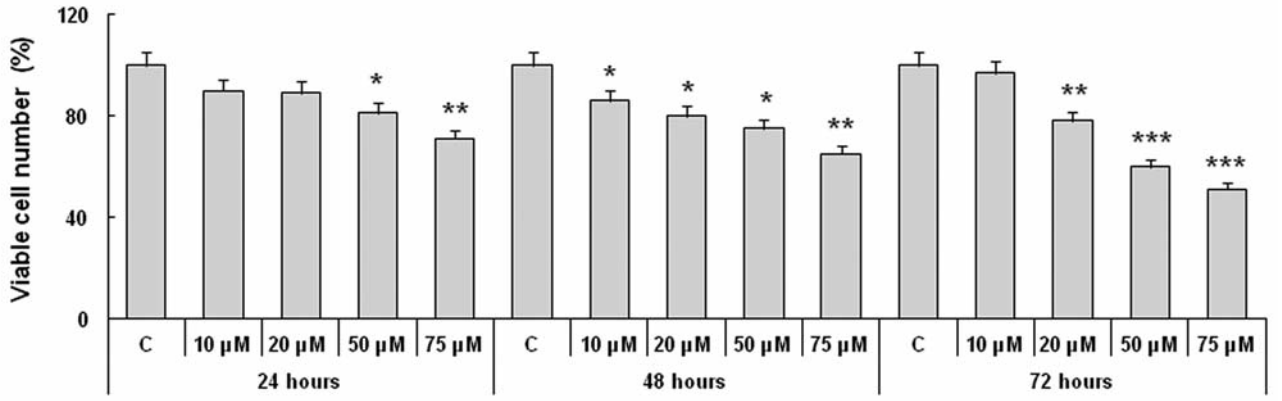

Figure 1. *Effect of NS398 on HT-29 cell growth. Exponentially-growing cells were seeded for 24 h in serum-free insulin-free medium and then treated with different concentrations of NS398 for 24, 48 or 72 h. Viable cell number was determined as the percentage of the corresponding control by the trypan blue exclusion test (n=6). Each bar represents the percentage (mean±S.D.) of three independent experiments, each performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 vs. control.*

molecular weight marker was also run simultaneously with the test samples. Images were digitalized by mean of VersaDoc Imaging System 4000 and analyzed with Quantity One software (Bio-Rad Laboratories, Milano, Italy).

Statistical analysis. Statistical significance of differences between independent groups were analyzed using the one-way ANOVA test with Bonferroni *post hoc* multiple comparisons. All values are expressed as means±SD, and differences were considered significant at $p<0.05$.

Results

Effects of EGF and NS398 upon HT-29 cell the proliferation under normoxic and hypoxic conditions. Since the medium containing 10% FBS is recognized to contain various types of growth factors including EGF, HT-29 cells were cultured in SFIF medium for 24 h before treatment. Under normoxic condition, treatment with increasing concentration (10-75 μM, final concentration) of COX2 inhibitor NS398 for 24, 48 and 72 h reduced viable cell numbers in a concentration- and time-dependent manner (Figure 1), with significant reductions occurring above all at 75 μM. To further determine the efficacy of NS398 against the stimulatory effect of EGF on HT-29 proliferation, the cells were also treated for 24 h with NS398 (20 and 75 μM) with/without EGF (100 ng/ml), under normoxic (Figure 2A) or hypoxic (Figure 2B) conditions, respectively. Cell counting revealed remarkable differences between cells cultured under normoxic and hypoxic conditions, since in hypoxic cells, the proliferative rate is higher. NS398 exhibited an inhibitory efficacy against EGF-induced cell proliferation only under normoxic conditions.

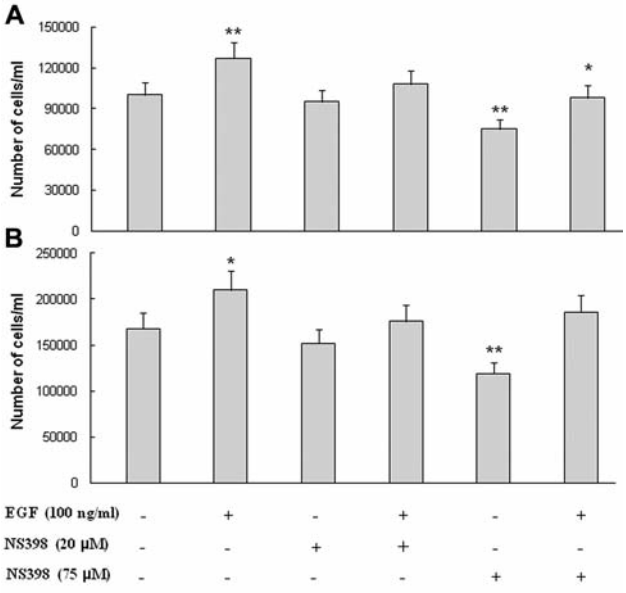

Figure 2. *Effect of epidermal growth factor (EGF) with/without NS398 on HT-29 cell proliferation under normoxia and hypoxia. Cells were incubated under normoxic (A) or hypoxic (B) conditions in serum-free insulin-free medium with the addition of EGF (100 ng/ml) and NS398 (20 and 75 μM). After 24 h, cells were harvested and their number was counted under a microscope. Each bar represents the number of cells/ml (mean±S.D.) for three independent experiments, each performed in triplicate. *p<0.05, **p<0.001 vs. respective control.*

Effects of EGF and NS398 on levels of proteins associated with proliferation. Exposure of HT-29 cells to recombinant human EGF induced a two-fold increase of COX2 levels and of phosphorylated forms of EGFR and ERK1/2, as well as higher levels of p-AKT and PI3K (Figure 3), compared to controls. In cells exposed to NS398, the levels of COX2, p-ERK1/2 and p-AKT decreased while p-EGFR levels were similar to those detected in control cells. Of interest, COX2

inhibitor NS398 was able to down-regulate EGF-induced increase of COX2, p-EGFR, p-ERK and p-AKT. Thus, to better-define the AKT pathway, we also examined the expression levels of *PTEN*, a tumour suppressor gene which acts as a negative regulator of PI3K/AKT pathway: results showed an increased expression (approximately 2-fold) in NS398-treated cells (more effectively at 20 μM), whereas exposure to EGF, both alone and in combination with COX2 inhibitor, did not substantially influence expression levels of the protein (Figure 3).

Modulatory role of hypoxia on HIF-1α and COX2-related pathways and on proteins downstream of EGFR signalling. To examine possible cross-talk between COX2 and HIF-1α pathways, we first investigated whether hypoxia affects COX2 expression over time. Western blotting of nuclear protein extracts showed that the maximal nuclear expression of HIF-1α, the key transcriptional regulator of cell response to hypoxia, was observed in cells cultured under hypoxia for 48 h. As far as COX2 expression is concerned, it is important to note that it was up-regulated by hypoxia and also remained elevated after 72 h under hypoxic conditions (Figure 4A).

In order to evaluate the role of hypoxia on COX2- and EGFR-related signaling pathway, HT-29 cells were treated for 24 h under hypoxic conditions with 75 μM NS398, the most effective concentration for COX2 inhibition, in the absence and presence of EGF. Interestingly, cell exposure to exogenous EGF resulted in elevated expression of HIF-1α (Figure 4B). More surprisingly, expression of HIF-1α was also increased after treatment with NS398, whereas concomitant treatment with EGF did not have any appreciable additional effects. It is important to note that cell exposure to EGF induced COX2 expression, effectively reduced by NS398.

In order to evaluate whether EGFR plays a pivotal role in the inhibitory effect of NS398 on hypoxic cells, expression of proteins downstream of EGFR signaling pathway were examined. In HT-29 cells cultured under hypoxic conditions, NS398 treatment resulted in an appreciable decrease of EGFR activation, especially in EGF-stimulated cells (Figure 4B). With regard to PI3K/AKT signaling pathway, the active, phosphorylated form of AKT was increased by EGF and, here again, the expression values were reduced when cells were treated with NS398 alone or in the presence of EGF (Figure 4B). In accordance to these results, in EGF-treated HT-29 cells, the expression of the tumour suppressor PTEN increased, as well as the levels of p-ERK and PI3K; NS398, both alone and in combination with EGF, was able to induce reduction of both expressions.

Effects of EGF with/without NS398 on EMT markers under hypoxia. HIFs are known to activate and regulate target genes involved in a number of different cellular processes linked to malignant progression, including EMT. It has been proposed that EMT can be counteracted by COX2 inhibition (23), thus we further analyzed at an early time point some critical parameters of hypoxia-induced EMT in the presence of COX2 inhibitor NS398 and EGF. E-Cadherin is a hallmark of EMT and, as shown in Figure 4C, treatment of HT-29 cells with EGF under 24 h hypoxia resulted in a mild reduction of E-cadherin levels and, respectively, up-regulation of β-catenin and N-cadherin expression. As far as exposure to NS398 is concerned, there was apparently no effect upon EGF-induced modulation of E-cadherin and N-cadherin expression, whereas EGF-dependent increase in β-catenin was inhibited.

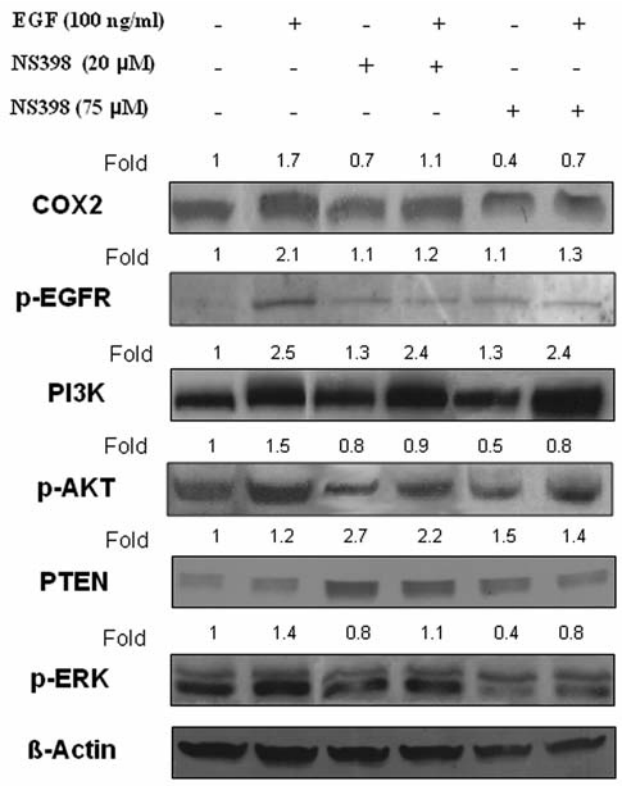

Figure 3. *Effect of epidermal growth factor (EGF) with/without NS398 on EGF receptor (EGFR) downstream signaling pathways under normoxic conditions. The cells were incubated for 24 h in the absence (control) or in the presence of EGF (100 ng/ml) and NS398 (20 or 75 μM), alone or in combination. Whole cell lysates were analyzed by western blotting with specific antibodies for cyclooxygenase-2 (COX2), p-EGFR, phosphatidylinositol-3-kinase (PI3K), p-protein kinase B (p-AKT), phosphatase and tensin homolog (PTEN) and extracellular-signal-regulated kinases 1/2 (ERK1/2) and then with horseradish peroxidase-conjugated secondary antibody. The optical density of the bands was determined by densitometry, normalized to β-actin, and expressed as arbitrary units relative to the control (S.D. <10%). The blots shown are representative of three independent experiments.*

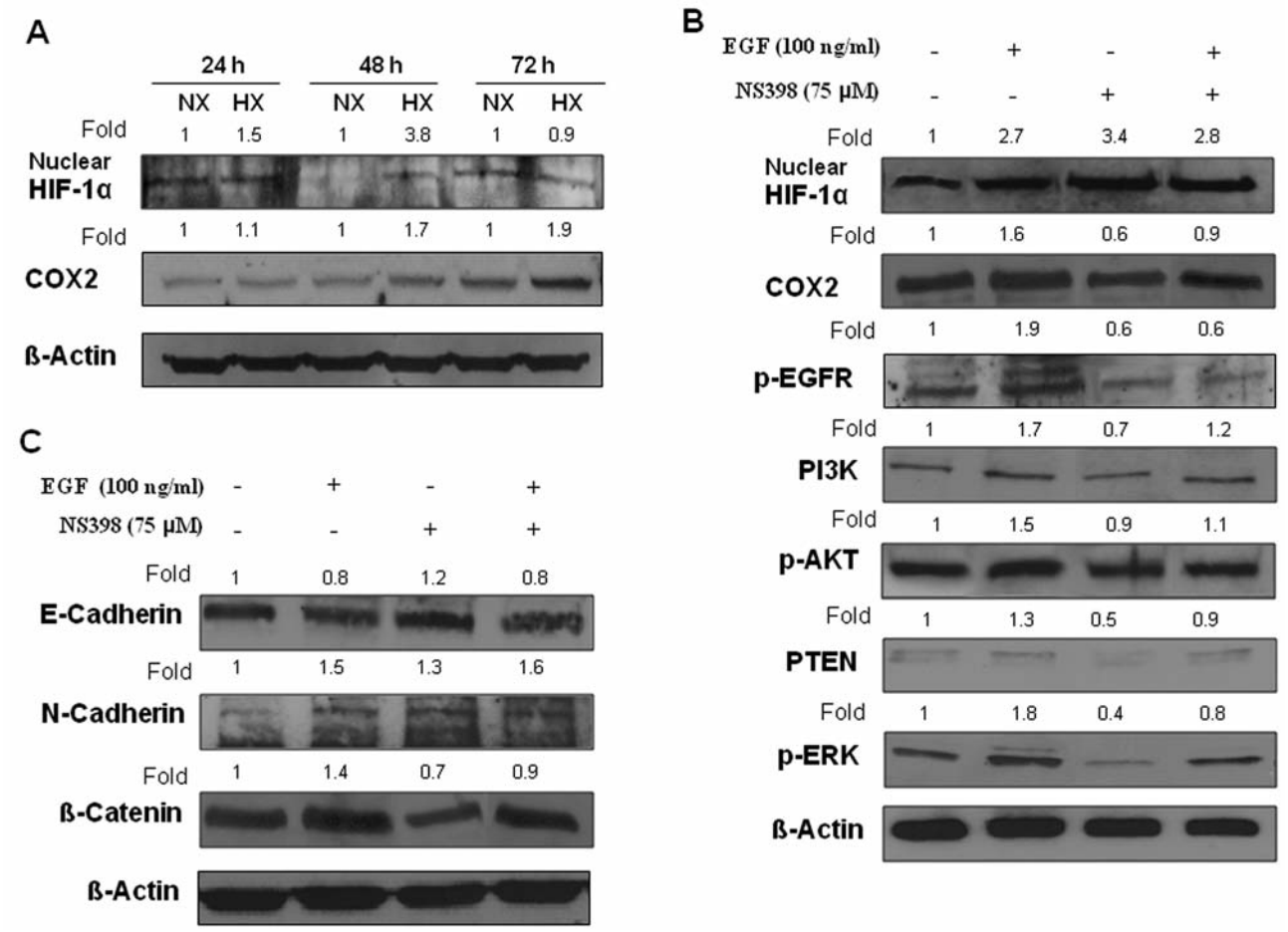

Figure 4. *Effect of hypoxia on hypoxia-inducible factor 1-alpha (HIF-1α), cyclooxygenase-2 (COX2) and epidermal growth factor receptor (EGFR)-related pathways and on epidermal-to-mesenchymal transition (EMT) markers following treatment with EGF with/without NS398. A: HT-29 cells were incubated for 24, 48 or 72 h under normoxic (NX) or hypoxic (HX) conditions. Nuclear and total cell lysates were analyzed by western blotting with specific antibodies for HIF-1α and COX2, respectively. B, C: HT-29 cells were treated for 24 h in the absence (control) or in the presence of EGF (100 ng/ml) and NS398 (75 μM), alone or in combination, under hypoxic conditions. Nuclear and total cell lysates were analyzed by western blotting with specific antibodies for HIF-1α, COX2, p-EGFR, phosphatidylinositol-3-kinase (PI3K), p-protein kinase B (p-AKT), phosphatase and tensin homolog (PTEN) and p-extracellular-signal-regulated kinases 1/2 (p-ERK1/2) (B) and for E-cadherin, N-cadherin and β-catenin (C). The optical density of the bands was determined by densitometry, normalized with respect to that of the corresponding β-actin band, and expressed as arbitrary units relative to the control (S.D. <10%). The blots shown are representative of three independent experiments.*

Changes in invasiveness mediated by NS398. EMT induction is strictly related to cancer cell invasiveness, and in order to investigate the effect of NS398 on HT-29 cells, the Boyden chamber-Matrigel invasion assay was employed, under normoxia and hypoxia (Figure 5A). We found that hypoxia strongly enhanced the highly invasive capacity of HT-29 cells, and that exogenous EGF markedly stimulated cell invasive ability, above all under normoxic conditions. Hypoxia-stimulated cell invasiveness was significantly reduced by treatment with NS398, whereas the COX2 inhibitor was more effective in reducing the invasive ability of cells that received EGF, whether cultured under normoxia or hypoxia.

Accumulating evidence has indicated that MMP2, as well as E-cadherin, are the target genes regulated by HIF-1α (24), thus MMP2 activity was examined by zymography. We observed that MMP2 release was significatively enhanced when cells were treated with EGF or cultured under hypoxia, and that the stimulatory effect was higher in hypoxic cells treated with EGF. After treatment with NS398, the activity of gelatinase MMP2 was reduced relative to the corresponding EGF-treated cells and corresponding hypoxic cells (Figure 5B). The enhancement in activity of MMP2 induced by combined EGF and hypoxia treatment was still inhibited by the COX2 inhibitor.

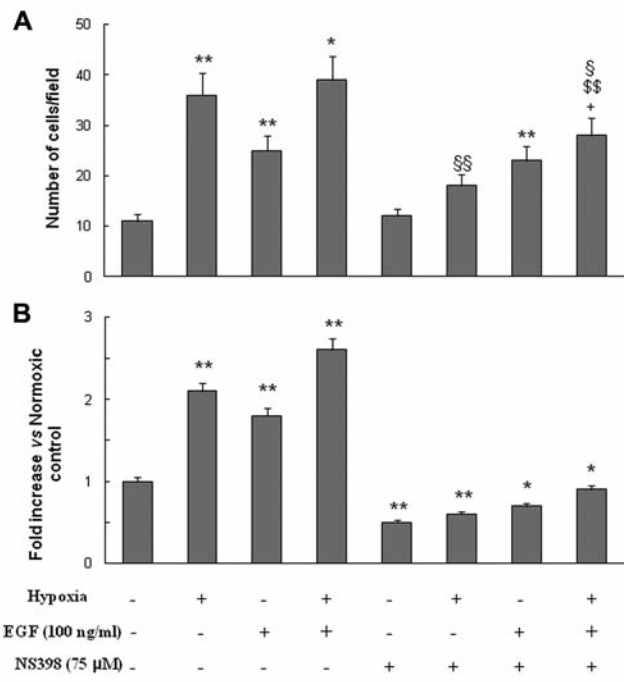

Figure 5. *Effects of epidermal growth factor (EGF) with/without NS398 on cell invasiveness under normoxia and hypoxia. HT-29 cells were incubated for 24 h under normoxic or hypoxic conditions in the absence (control) or in the presence of EGF (100 ng/ml) and NS398 (75 μM), alone or in combination. A: Matrigel invasion was evaluated by employing Boyden chamber's assay; each filter was examined with a Zeiss microscope (×400 final magnification) and the number of cells able to invade the matrigel was counted. Data are expressed as the number of cells per high-magnification field and represent the mean of three independent experiments, each performed in triplicate (bars, S.D.). *p<0.05, **p<0.001 vs. normoxic control; §p<0.05, §§p<0.001 vs. hypoxic control; $$p<0.001 vs. NS398-treated hypoxic cells; + p<0.05 vs. EGF-treated normoxic cells. B: Matrix metalloproteinases-2 (MMP2) release was determined by gelatin zymography of cell-free supernatants. The zymograms shown are representative of three independent experiments. Gelatinase activity was estimated by densitometry and expressed as arbitrary units relative to normoxic control (bars, S.D.). *p<0.01, **p<0.001 vs. normoxic control.*

Discussion

The present work aimed to investigate the role of tumour microenvironmental factors such as EGF and hypoxia on the contribution of COX2 to colon cancer cell aggressiveness. Clinical and experimental data have indicated that increased COX2 expression correlates with aggressive growth characteristics and poor prognosis for patients with high-grade carcinomas (25, 26). COX2 is also elevated in response to hypoxia, and it has been reported that the hypoxia-induced HIF-1α protein, considered as a master transcription factor regulating multiple genes involved in tumour invasion (27), interacts with the HIF-responsive elements in the COX2 promoter, thereby inducing its expression. EGFR signaling is another key regulator of colorectal carcinogenesis, and, similarly to COX2, high levels of EGFR expression have been associated with poor prognosis and diminished overall survival of patients with colon cancer (4, 28).

The existence of a relationship between COX2 and EGFR is well-known, since COX2 has been identified as a putative EGF target gene, and COX2-derived PGE_2 has been reported to transactivate EGFR (29). We observed that in HT-29 colon cancer cells, EGF increased COX2 protein expression, in accordance with other authors who demonstrated that after EGF stimulation of CRC cells, COX2 expression increased through activation of EGF-mediated signaling pathways (29, 30). The major intracellular means of transducing the signals elicited by EGF are ERK and PI3K/AKT signalling pathways, and here we showed that cell treatment with COX2 inhibitor NS398 resulted in a significant decrease of phosphorylation of ERK and downstream phosphoproteins of the PI3K/AKT pathway. Thus, we suggest that EGF promotes HT-29 colon cancer cell growth partly through COX2-related PI3K/AKT signaling pathway, which plays critical roles in mammalian cell survival and resistance to apoptosis (31). Higher expression and activity of AKT is essential for cell survival during tumorigenesis, and its aberrant activation is a result of dysregulation in the oncogenic PI3K/phosphatase (32). Activation of AKT by PI3K is negatively-regulated by *PTEN*, a critical tumor suppressor gene often mutated or silenced in human cancer (33). PTEN regulates multiple cellular processes such as cell proliferation, survival, growth, and motility, principally by inhibiting PI3K/AKT signalling. Some authors also demonstrated that certain non-steroidal anti-inflammatory drugs, such as sulindac and celecoxib, enhanced PTEN levels (34), thus regulating AKT levels during the early neoplastic transformation of colon, and our results show that the COX2 inhibitor increased PTEN expression in HT-29 cells under normoxic conditions, but failed to do the same under hypoxia. A hypoxic tumor microenvironment contributes to cancer progression through induction of HIF-1α, which is activated and expressed in the majority of human colon carcinomas (8). Under oxygen-deprived conditions, increased levels of HIF-1α are due to reduction of proteasomal degradation resulting from the inactivation of HIF prolyl-hydroxylase. Previous studies reported that under hypoxic conditions, COX2 is induced by HIF-1α (35); on the other hand, an elevated level of PGE_2 in hypoxic colon tumour cells enhances transcriptional activity of HIF-1α by activating the MAPK pathways and, as a consequence, COX2 selective inhibitors would reduce HIF-1α transcriptional activity. In contrast with these reports, here we showed that the COX2 inhibitor NS398 seemed to rather induce HIF-1α expression, also strengthened by EGF; thus it is conceivable that in HT-29 cells, NS398 can lead to HIF-1α expression through activation of EGFR.

We found that NS398 reduced basal EGFR activation/phosphorylation above all in hypoxic cells, suggesting that, similarly to celecoxib and other anticancer agents that have been proposed to exert their anti-proliferative effects by promoting degradation or internalization of EGFR (36), its effects on EGFR downstream signalling pathways may rely on mechanisms of action related to HIF1-α stabilization rather than to COX2 inhibition.

HIF-1α is localized in the cytoplasm or nuclei of various human colon cancer cells (10), and its stabilization and activation correlates with various stages of tumor progression also through the activation of genes involved in tumor cell invasiveness. Up-regulation of MMPs contributes to ECM remodelling, accelerating cell migration and invasion (11). In colon carcinomas, both MMP2 and MMP9 have been found to be overexpressed at the mRNA level. HIF-1α could thus mediate hypoxia up-regulation of MMPs, suggesting that it is highly important to modulate the expression and activity of HIF-1α, in order to have the potential to improve the efficiency of clinical treatment of cancer. On this regard, our findings suggest that COX2 inhibition may impair the complex multi-step process associated with increased cell motility and lysis of the ECM also through down-regulating the expression of MMP2. A major mechanism which is likely to link hypoxia to cancer progression is further represented by the expression of proteins that favour tumor invasiveness through adaptive mechanisms involving specific HIFs and resulting in the induction of EMT (22), in which the loss of epithelial characteristics causes dissociation from surrounding cells and acquisition of mesenchymal-like properties, which allows cancer cells to migrate away from the initial neoplastic focus. EMT is regulated by signals coming from the tumor microenvironment, including hypoxia and EGF. In addition, tumours themselves can release growth factors and proteases, thus modifying the tumour microenvironment and favouring cell invasiveness. Previously we demonstrated that the selective COX2 inhibitor celecoxib blocked EMT induced by hypoxia and EGF (22) in HT-29 cells. In the present study, we also found that NS398 is highly effective in preventing invasive activity induced by hypoxia and EGF signalling, thus confirming that COX2 inhibition may represent a mechanistic link between hypoxia and EMT.

Conclusion

The relationship between microenvironmental factors and COX2 may impact on colon tumor malignancy, and these findings may offer additional insights into mechanisms that govern COX2/EGFR regulation in growth and invasion of colon cancer. Taken together our data indicate that hypoxia is a key regulator of COX2 expression and cellular features in colon epithelial cells, acting in concert with EGFR through a PI3K/AKT-dependent mechanism. Thus, our observations suggest the possibility that COX2, as well as EGFR, may be potential therapeutic targets that, in combination with anti-hypoxia therapies, could be considered in the development of novel treatments for colon cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Acknowledgements

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