Original Paper

Cadmium Induces Migration of Colon Cancer Cells: Roles of Reactive Oxygen Species, P38 and Cyclooxygenase-2

Sara Naji[a]   Khodr Issa[a]   Assaad Eid[b]   Rabah Iratni[c]   Ali H. Eid[a,d]

[a]Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon, [b]Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon, [c]Department of Biology, College of Science, United Arab Emirates University, Al-Ain, United Arab Emirates, [d]Department of Biomedical Sciences, College of Health Sciences, Qatar University, Doha, Qatar

Abstract

Background/Aims: Cadmium (Cd) is a heavy metal contaminant whose toxicity is associated with colorectal cancer (CRC). However, the underlying molecular mechanisms of Cd-induced CRC malignancy remain obscure. **Methods:** A monolayer scratch assay was employed to assess the migration of HT-29 human adenocarcinoma cells. Luciferase reporter assay was used to determine cyclooxygenase-2 (COX-2) transcriptional activity, and Western blotting was used to detect p38 Mitogen Activated Protein Kinase (MAPK) and Akt phosphorylation as well as COX-2 expression. Prostaglandin E_2 (PGE_2) levels were measured using Enzyme Linked Immunosorbent Assay (ELISA) and reactive oxygen species (ROS) formation was assessed using dihydroethidium (DHE) stain. **Results:** Here, we show that Cd potentiates the migratory capacity of HT-29 CRC cells. Cd caused a time-dependent increase in COX-2 expression. Celecoxib, a COX-2 selective inhibitor, significantly reduced Cd-induced migration. Cd also increased levels of ROS and phosphorylated p38. Importantly, Cd-induced COX-2 expression and migration were significantly abolished by N-Acetyl-Cysteine (NAC), a ROS scavenger, or SB202190, a specific p38 inhibitor. Furthermore, Cd-induced p38 phosphorylation was inhibited by NAC. Cd (100 nM) also increased PGE_2 levels, which was abrogated by NAC, SB202190, or celecoxib. Exogenous PGE_2 significantly potentiated cell migration. Cd caused a significant increase in Akt phosphorylation in a ROS-mediated pathway. Moreover, Cd-induced migration was significantly attenuated by LY294 002, a phosphatidylinositol-3-kinase inhibitor. **Conclusion:** Taken together, our results suggest that exposure to low levels of Cd promotes a more migratory cancer phenotype in a ROS-p38-COX-2-PGE_2 pathway as well as ROS-Akt pathway. Therefore, COX-2, PGE_2 receptors or Akt represent potential targets in the treatment of CRC, particularly in patients exposed to Cd.

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Dr. Ali H. Eid

Department of Pharmacology and Toxicology, Faculty of Medicine, American University of Beirut; Bliss Street, Riad El Solh, Beirut 1107-2020 (Lebanon)
Tel. +961-1-350-000, Fax +961-1-343-450, E-Mail ae81@aub.edu.lb

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Introduction

Colorectal cancer (CRC) is currently the second leading cause of cancer related deaths in the world [1]. Risk factors associated with CRC are mainly divided into the modifiable (obesity, physical inactivity, diet, smoking) and the non-modifiable ones (age, gender, family history) [2]. Recently, the exposure to environmental pollutants such as heavy metals has gained support as possible contributors to increased CRC risk. These metal contaminants generally include: Arsenic, lead, mercury, chromium and cadmium [3, 4].

Cadmium (Cd) is a toxic metal present in the environment and used in industrial activities [4, 5]. Accumulating evidence suggests that Cd is associated with a wide range of adverse health effects such as cardiovascular diseases [6], bone-related disorders [7], reproductive toxicity [8], nephrotoxicity [9], inflammatory diseases, and cancer [10]. In fact, Cd is classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC). The exact molecular mechanisms by which Cd promotes malignancy are poorly understood [11]. Nevertheless, it has been postulated that Cd-induced reactive oxygen species (ROS) formation, by activating multiple signaling players such as the Mitogen activated protein kinases (MAPKs) [12], play a key role in Cd-mediated carcinogenicity [11, 13].

Different cancer types have been linked to Cd exposure. Being a metalloestrogen, Cd was reported to mimic the actions of estrogen in biological systems. It is not surprising then that Cd has been positively associated with hormone-dependent cancers such as endometrial [14] and breast cancers [15]. It has been suggested that Cd targets the prostate gland as well [16]. *In vitro* studies indicate that Cd promotes the transformation of normal human prostate epithelial cells to a malignant phenotype [17, 18]. Recent evidence shows that dietary Cd exposure contributes to the development of prostate cancer [16]. Contextually, CRC is among the different types of cancers associated with Cd. A significantly higher Cd plasma level was reported in metastatic CRC patients when compared to healthy group [19]. Experimental evidence further demonstrates that by suppressing DNA mismatch repair mechanism, Cd acts a mutagen [20] and might therefore contribute to CRC. Moreover, Cd was shown to induce transformative and carcinogenic effects in human colorectal cells CRL-1807 and a xenograft animal model [21].

Inflammation has been also linked to Cd exposure [22]. In fact, inflammatory bowel diseases (IBD) are among those that have been positively correlated with Cd. Oral Cd exposure in rats was reported to increase the severity of colonic inflammation and to delay the healing of acetic acid-induced colitis [23]. Furthermore, evidence indicates that Cd initiates a pro-inflammatory response by inducing cyclooxygenase -2 (COX-2) upregulation in mouse neuronal cells [24].

Substantial evidence indicates that inflammation is strongly correlated with the initiation and development of cancer [25, 26]. In fact, persistent inflammation as a result of infection or injury could predispose to cancer development. For instance, chronic infections as in hepatitis B or C are associated with elevated hepatocellular carcinoma risk [25]. Moreover, chronic inflammation of intestine as in patients with IBD is strongly linked to increased CRC risk [27]. The cyclooxygenase (COX) pathway is among those inflammatory pathways that have been suggested to play a central role in colorectal malignancy. The COX enzyme exists in two major isoforms: COX-1, the constitutive enzyme and COX-2 the inducible one [28]. It is important to note that both enzymes serve as molecular targets for the Non-steroidal Anti-inflammatory drugs (NSAIDs), a class of medications used in the management of pain, fever and inflammation.

Overwhelming data suggests that COX-2 is positively correlated with colorectal malignancy. COX-2, but not COX-1, gene expression was reported to be highly elevated in human CRC mucosa when compared to the normal one [29]. Moreover, COX-2 overexpression was found to be associated with worse survival among CRC patients [30]. The regular use of aspirin (an NSAID) after diagnosis with CRC is linked to a lower CRC specific and

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overall mortality [31]. Experimental evidence demonstrates that selective COX-2 inhibitors negatively regulate CRC cells growth, migration and invasion [32, 33].

The tumorigenic effects of COX-2 are thought to be largely attributed to its major metabolite, Prostaglandin E_2 (PGE_2). In fact, elevated PGE_2 levels was reported among patients with colonic adenomas as well as carcinoma [34]. The biological functions of PGE_2 are meditated by binding to its target receptors, namely the E type prostanoid (EP) receptors [35]. These receptors comprise the following EP1, EP2, EP3 and EP4 subtypes. Recent evidence suggests that the EP receptors, namely EP4, play a central role in colorectal neoplasia [36]. PGE_2 exerts a plethora of effects in colorectal cancer promoting proliferation [37], survival, angiogenesis [38], invasion and migration [39, 40] . Multiple players were shown to be implicated in COX-2/PGE_2 mediated carcinogenicity including the Phosphatidylinositol 3-kinase (PI3K)/Akt pathway. For instance, PGE_2 via activating EP4 receptors, increases the growth, migration and invasion of LS-174T colorectal carcinoma cells in a PI3K/Akt mediated pathway [41].

Despite being a known human carcinogen, the underlying molecular mechanisms of Cd-induced colorectal malignancy remain vague. Thus, in the present study we hypothesize that Cd promotes the migration of CRC cells by increasing PGE_2 production via a ROS-p38 MAPK-COX-2 dependent mechanism, concomitant with activation of the ROS-Akt pathway.

Materials and Methods

Reagents

Cadmium Chloride dihydrate ($CdCl_2$) was a kind gift from the chemistry department at American University of Beirut (AUB). Rabbit monoclonal anti-COX-2 (ab15191), anti-β actin (ab119716), anti-p38 (phospho T180+Y182 ab38238) anti-total p38 (ab7952), anti-pan AKT (ab8805), anti-pan AKT (phospho T308 ab38449), SB202190 (ab20638) and Celecoxib (ab141988) were purchased from abcam. Goat anti-rabbit secondary antibody IgG (H+L), HRP conjugate was obtained from Invitrogen. N-Acetyl-Cysteine (#1761C311) was obtained from Amresco (Solon, Ohio, USA). LY294 002, Dulbecco's Modified Eagle's medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich (Schnelldorf, Germany) and PGE_2 was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Penicillin/streptomycin was obtained from Lonza (Basel, Switzerland).

Cell culture

HT-29 human colorectal adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown in 10 mm dishes and maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Culture medium was changed every 48 hours and cells were split in a ratio of 1 to 4 once reaching 90% confluence.

Migration Assay

Cell migratory capacity was assessed using the scratch or wound healing assay. Cells were seeded in 12-wells plate ($6x10^5$ cells/well) in 1 ml complete DMEM. Once reaching 90-95% confluence, a scratch was created manually through the confluent monolayer using a white tip (2–20 μl). The culture medium was then aspirated, and wells were washed with PBS to remove cellular debris. Complete fresh medium was then added along with the indicated treatments. Photomicrographs were taken at baseline (0 hour) and at the indicated time points. ZEN lite from Zeiss Microscope software was used to measure the width of the scratch which reflected the extent of cell migration. The distance migrated was measured in μm.

Western blotting

Cells were lysed using a lysis buffer comprising 10 mM Tris pH 6.8, 2% SDS, as we previously described [42]. Proteins were quantified by DC protein assay kit (Bio-Rad, USA). Protein samples were then loaded (30-50 μg) on 5-11% SDS-PAGE along with protein ladder (Abcam), electrophoresed at 70 V (Bio-Rad, USA) and then transferred to PVDF membranes (Bio-Rad, USA). After blocking with 5% non-fat dry milk in TBS-T (TBS and 0.05% Tween 20) for 1 hour at room temperature, the blots were probed with

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primary antibody at 4°C overnight. Blots were then washed three times with TBS-T and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:2000) for 1 hour at room temperature. Immunoreactive bands were finally detected by ECL chemiluminescent substrate (Bio-Rad, USA). Blot images were taken by using Chemidoc MP Imaging system (Bio-Rad, USA).

Dihydroethidium staining (DHE)

ROS production was assessed using DHE stain. HT-29 cells were seeded on a cover slip in 12-well plates (3×10^5 cells/well) in 1 ml complete DMEM and incubated until reaching 50% confluency. The cells were treated as indicated. Medium was then removed, cells were washed twice with cold PBS. DHE stain (5 μM final concentration) was added to the wells (1ml/well) in the dark and cells were incubated for 1 hour. DHE stain was removed and cells were washed once with cold PBS. Stained cells on the cover slips were imaged using Microscope Zeiss Axio.

Transient Transfections

COX-2 transcriptional activity was measured using the luciferase reporter assay. Cells were transiently transfected with COX-2-promoter driven luciferase expression reporter plasmid using Lipofectamine 2000, to achieve an approximate 80% transfection efficiency. Cells were then allowed to recover overnight in complete growth medium. The next day, cells were treated as indicated. For the luciferase analysis, cells were washed, lysed in luciferase lysis buffer (Promega; Wisconsin, USA), snap-frozen, and then thawed at room temperature. This was followed by the centrifugation of the cell lysates at 9, 300 g for 10 minutes. The luciferase activity was finally determined in the supernatant.

Enzyme linked immunosorbent assay (ELISA)

HT-29 cells were subjected to the specified treatment conditions for the indicated time points and PGE_2 production was evaluated using the PGE_2 Enzyme linked immunosorbent assay (ELISA) kit (R&D Systems; Minnesota, USA) according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed by a student's t-test for either paired or unpaired observations using GraphPad Prism version 5.0 and InStat3 Software (GraphPad software, Inc. San Diego, CA). Data was presented as mean ± SEM, where n is equal to the number of times an experiment is repeated (n will be = 3). When more than two means are used for comparison, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). $p < 0.05$ is considered significant.

Results

Cd induces the migration of HT-29 cells

Cd, at a concentration of 100 nM, significantly ($p < 0.05$) promoted wound healing at 9 and 12 hours (Fig. 1A and B). The 1000 nM concentration, on the other hand, significantly ($p < 0.05$) increased the migration only at 9 hours (Fig. 1B), and no further increase was observed for prolonged time.

Cd increases colon cancer migration through production of reactive oxygen species

Because Cd was previously shown to induce ROS accumulation in various cells, we therefore investigated its effect on ROS production in HT-29 colon cancer cells. As shown in Fig. 2C and 2D treatment significantly increased ROS production as early as 5 minutes.

We next examined the effect of ROS production on the Cd-induced migration. We found that pre-treatment of HT-29 cells with the ROS scavenger N-Acetyl-Cysteine, NAC (10 nM), for 30 minutes significantly ($p < 0.05$) attenuated Cd-induced migration (Fig. 3A and B).

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Fig. 1. Cd increases the migration of HT-29 cells. Cell migration was examined by the wound-healing assay. HT-29 cells were treated with Cd (100 and 1000 nM). Images were taken at the indicated time points. Values are represented as mean ± SEM of n=3. * denotes p<0.05.

Fig. 2. Cd increases the production of ROS. HT-29 cells were treated with Cd (100 nM) for 5, 10, and 30 minutes and DHE staining assay was performed.

Cd promotes colon cancer cell migration through ROS-dependent activation of p38 MAPK

Next, we have investigated whether the p38 MAPK pathway is implicated in the Cd-mediated effect on cellular migration, by measuring the level of p38 phosphorylation. We found that Cd caused more than 2-fold increase in p38 phosphorylation (p < 0.05) following 10 and 30 minutes of treatment (Fig. 4A and B). Furthermore, pretreating the cells with SB202190 (10 µM), a specific p38 MAPK inhibitor, significantly (p < 0.05) abrogated Cd-induced migration (Fig. 4C and D).
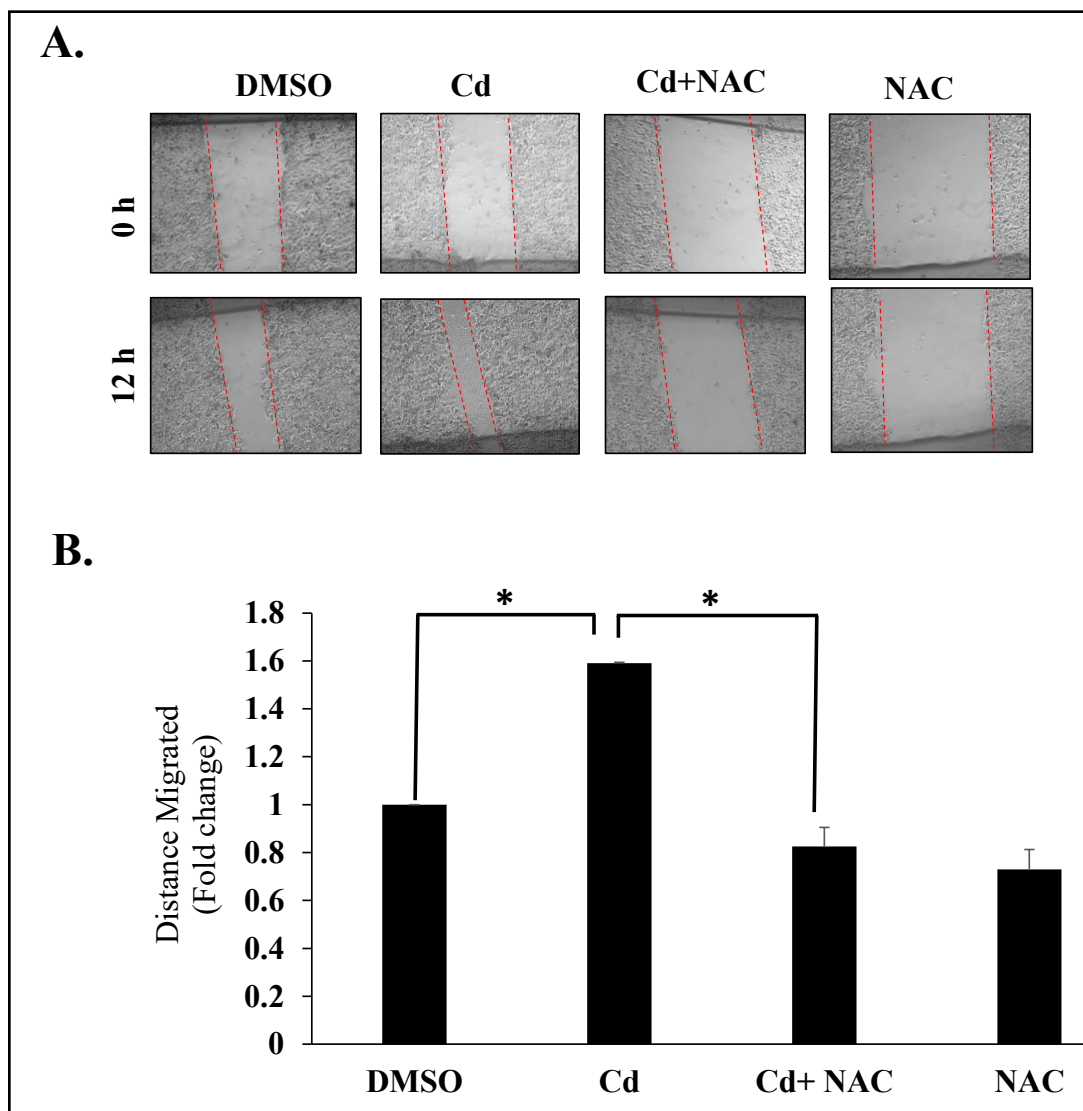
Fig. 3. Cd-induced ROS production increases HT-29 migration. HT-29 cells were treated with Cd (100 nM) in the presence or absence of the ROS scavenger NAC (10 mM) and migration was assessed by scratch assay. Values are represented as mean ± SEM of n=3. * denotes p<0.05.

Next, we examined whether the Cd-induced activation of p38 MAPK is mediated by ROS production. Indeed, we found that pre-treatment with NAC significantly (p < 0.05) abolished Cd-induced p38 activation (Fig. 5A and B).

Cd increases COX-2 expression at the transcriptional and protein levels
Cd significantly (p < 0.05) increases COX-2 protein levels as early as 6 hours (Fig. 6A and B) and maximally at 24 hours (Fig. 6C and D). This result was further validated by assessing the effect of 100 nM Cd on COX-2 expression at the transcription level. Consistent with the protein results, Cd caused a significant and time-dependent increase (p < 0.05) in transcriptional activity of COX-2 promoter, with this increase being evident as early as 6 hours after treatment (Fig. 6 E).
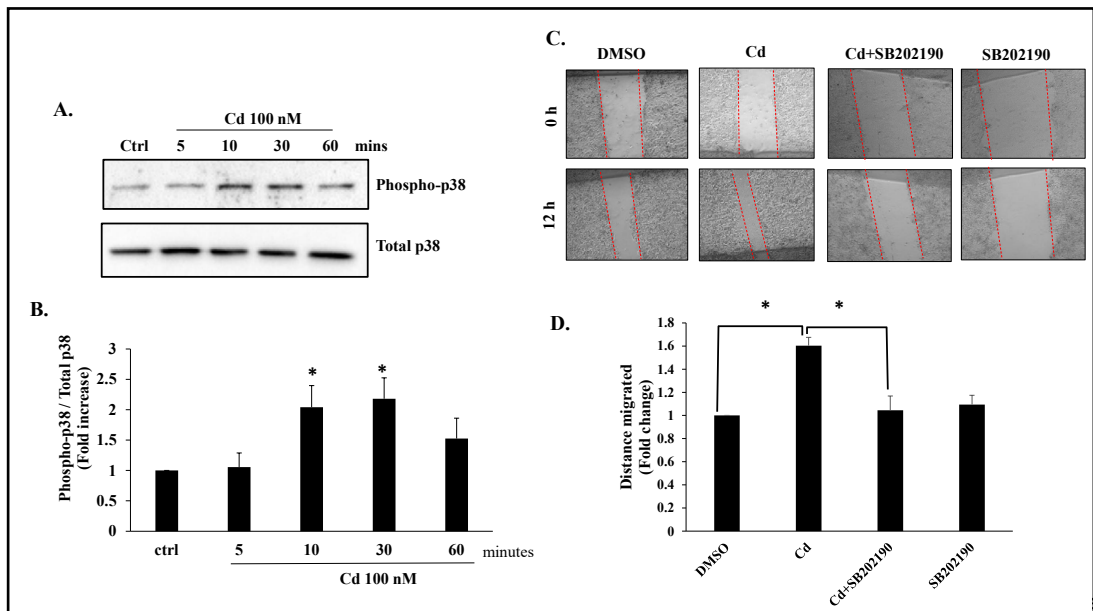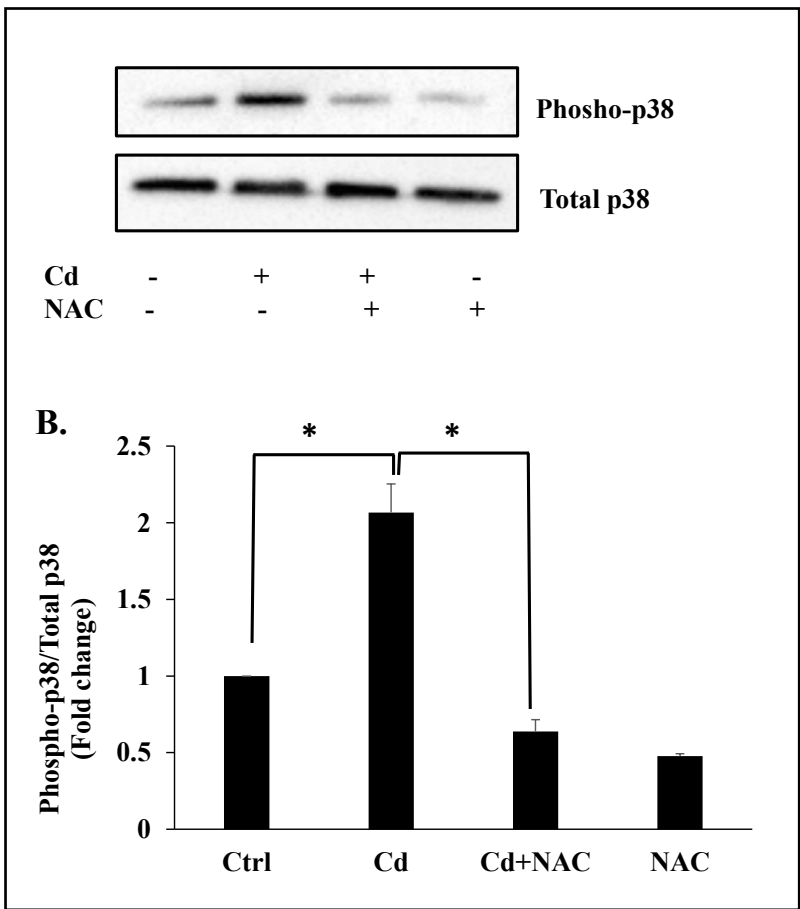
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Fig. 4. Cd activates p38 MAPK which is implicated in migration. (A) and (B): HT-29 cells were treated with Cd (100 nM) for 0, 5, 10, 30 and 60 minutes and p38 MAPK phosphorylation levels were determined by western blotting. (C) and (D): Cells were pretreated with SB202190 (10 µM) for 30 minutes followed by Cd (100 nM) and migration was determined by wound healing assay. Values are the mean ± SEM of three replicates. * denotes $p < 0.05$.

Fig. 5. Cd-induced ROS is upstream p38 MAPK. HT-29 cells were treated with Cd (100 nM) for 30 minutes in the presence or absence of NAC and p38 MAPK phosphorylation levels were determined by western blotting. Values are the mean ± SEM of three replicates. * denotes $p < 0.05$.
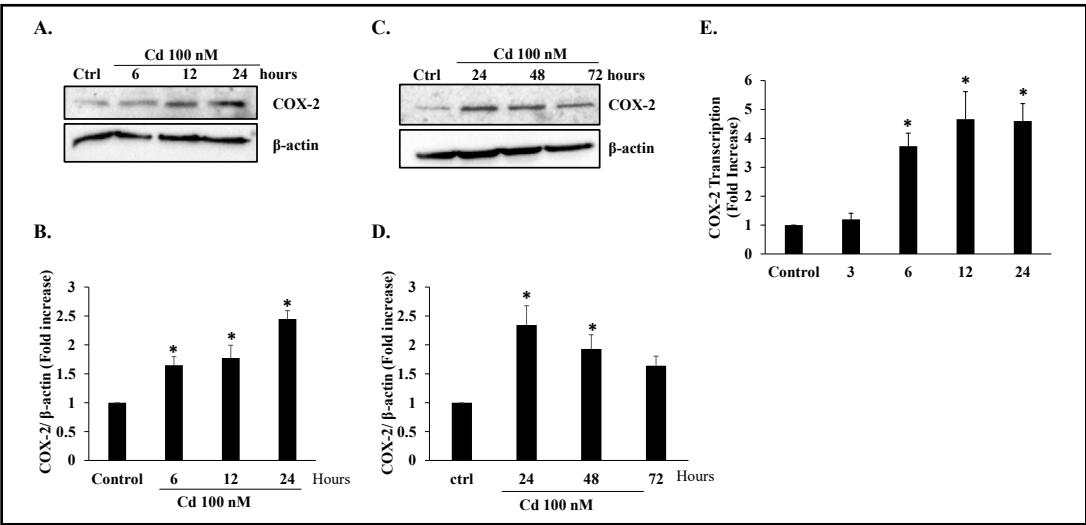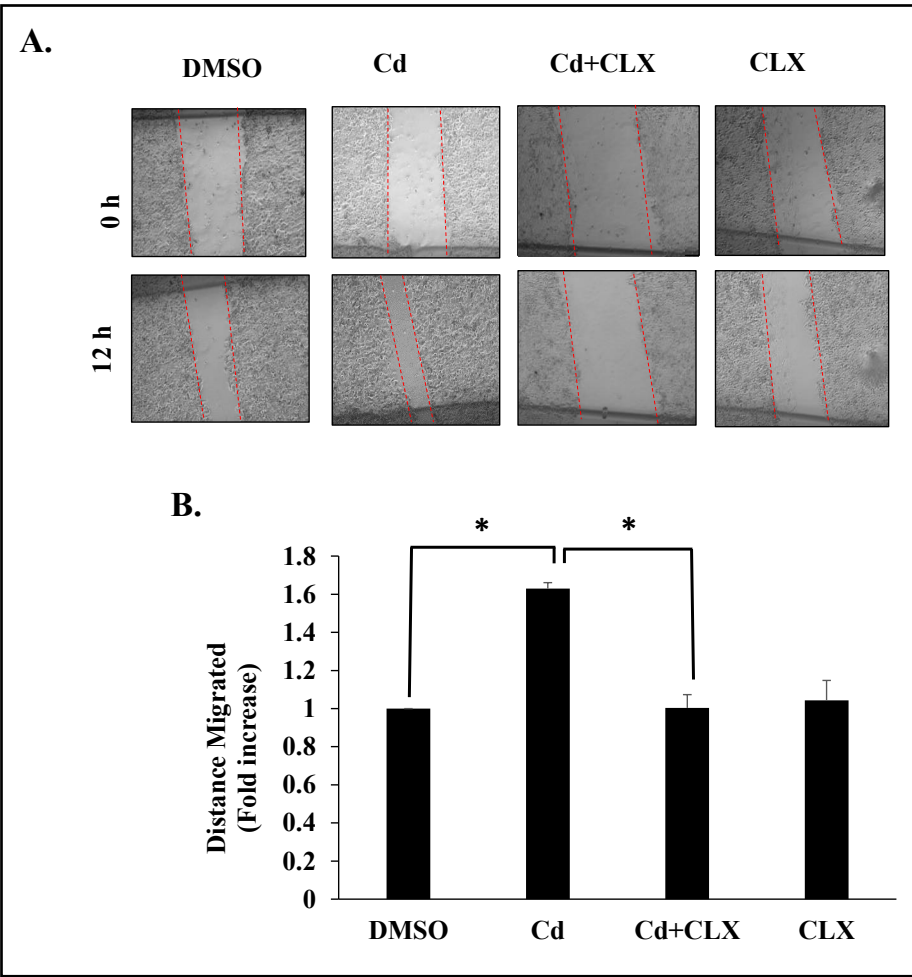
Fig. 6. COX-2 protein expression and transcription are enhanced in response to Cd. (A), (B), (C) and (D): HT-29 cells were treated with Cd (100 nM) for 6, 12, 24, 48 and 72 hours. COX-2 protein expression was assessed by western blotting. (E): Cells were treated with Cd (100 nM) for 3,6,12 and 24 hours. COX-2 transcription level was assessed by measuring the level of COX-2 promoter-driven luciferase using reporter assay. Values are the mean ± SEM of three independent experiments. * denotes $p<0.05$.

Fig. 7. Cd-induced migration is COX-2 dependent. HT-29 cells were pre-treated with CLX (10 µM) for 30 minutes followed by Cd (100 nM). Migration was determined by wound healing assay. Values are represented as mean ± SEM of n=3. * denotes $p<0.05$.

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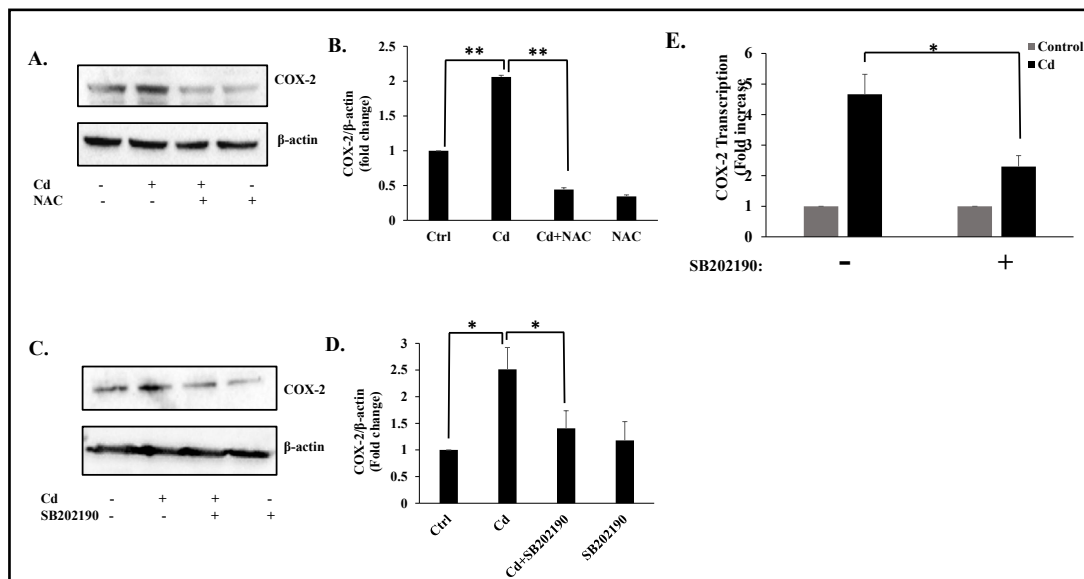Naii et al.: Cadmium and Colorectal Cancer

Fig. 8. Cd acts through ROS and p38 to upregulate COX-2. (A), (B), (C) and (D): HT-29 cells were treated with Cd (100 nM) for 24 hours in the presence or absence of NAC and/or SB202190. COX-2 protein levels were assessed by western blotting. (E): Cells were pre-treated with SB202190 for 30 minutes followed by Cd (100 nM) for 12 hours. COX-2 transcription was evaluated using the COX-2 promoter-driven luciferase reporter activity. Values are represented as mean ± SEM of n=3. * denotes $p<0.05$, ** denotes $p<0.01$.
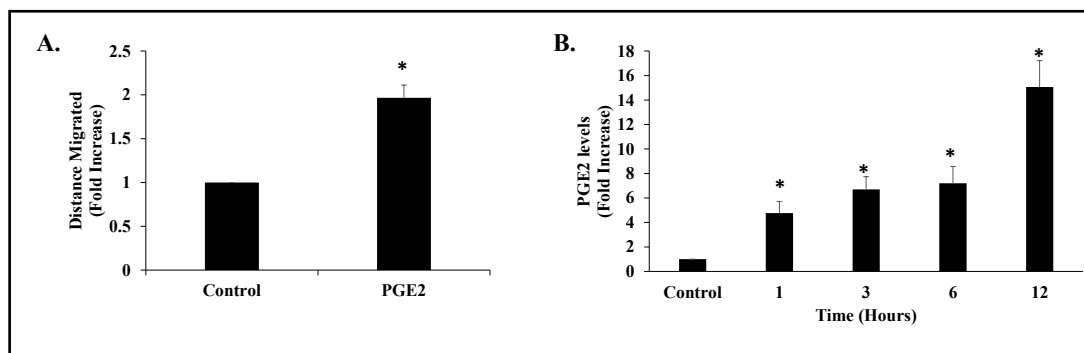

Fig. 9. PGE_2 enhances migration and its levels are increased in response to Cd. (A): Cells were treated with 1 µM PGE_2 for 12 hours and migration was assessed by scratch assay. (B): Cells were treated with Cd (100 nM) for 1, 3, 6 and 12 hours and PGE_2 production was assessed using ELISA kit. Values are represented as the mean of fold increase ± SEM of three independent experiments. * denotes $p<0.05$.

Cd-induced migration is mediated by ROS-dependent p38 MAPK through upregulation of COX-2

Next, we wanted to examine whether COX-2 induction is involved in Cd-induced migration of colon cancer cells. As shown in Fig. 7A and 7B, selective inhibition of COX-2 with celecoxib (CLX; 10 µM) significantly ($p <0.05$) abolished Cd-induced migration, hence suggesting that Cd induced migration requires COX-2 function.

Next, we sought to investigate the mechanism through which Cd treatment upregulates COX-2 expression. As shown in Fig. 8, Inhibition of ROS production by NAC (Fig. 8A and B) and inhibition of p38 (Fig. 8C and D), by SB202190, significantly attenuated Cd-induced upregulation of COX-2 protein. Moreover, pretreatment with SB202190 also resulted in a significant ($p < 0.05$) reduction in Cd-induced increase in the transcription level of COX-2 at 12 hours (Fig. 8 E).
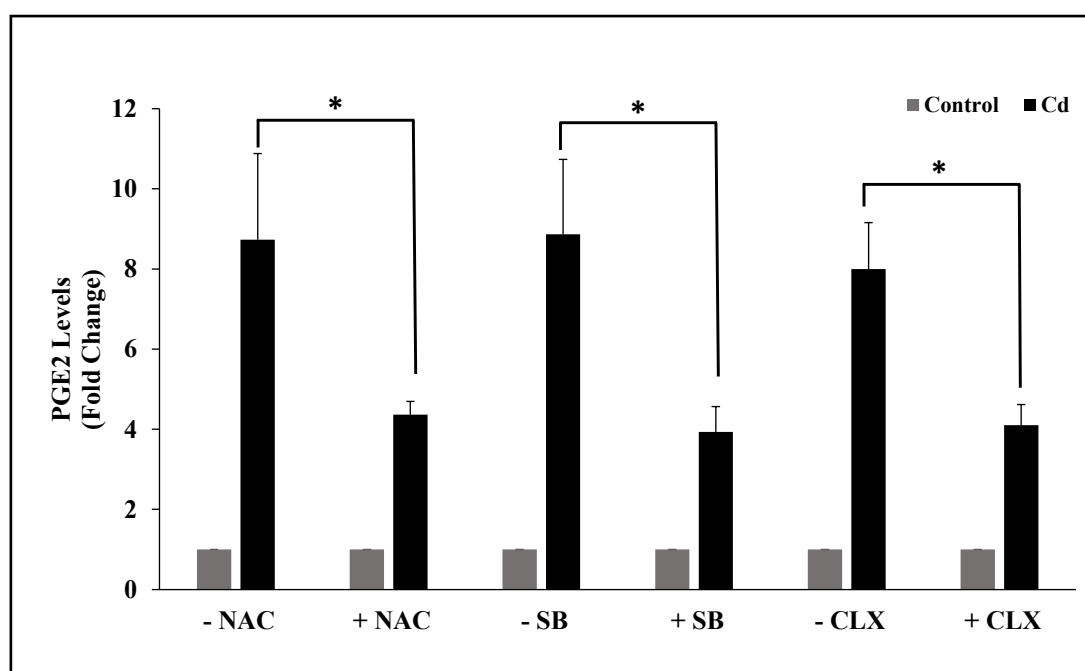
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Fig. 10. Cd increases PGE_2 levels in a ROS, p38 and COX-2 dependent pathway. HT-29 cells were pre-treated with NAC, SB202190 or CLX for 30 minutes followed by Cd (100 nM) for 6 hours. PGE_2 levels were measured using ELISA kit. Values are represented as the mean of fold change ± SEM of n=3. * denotes $p<0.05$.

PGE_2 potentiates migration and its production is enhanced in response to Cd through ROS mediated activation of p38MAPK and subsequent COX-2 upregulation

We have shown that COX-2 is required for Cd-induced migration of colon cancer cells (Fig. 7A and B). We therefore investigated whether Prostaglandin E_2 (PGE_2), a COX-2 major metabolite, is involved in the increased migration of Cd-treated HT-29 cells. We found that exogenous PGE_2 significantly ($p < 0.05$) increased HT-29 migration (Fig. 9A). Moreover, Cd caused a concentration-dependent increase in endogenous PGE_2 production (data not shown). Time-course experiment showed that Cd (100 nM) caused a time-dependent increase ($p <0.05$) in PGE_2 production starting as early as 1 hour (Fig. 9B).

Interstingly, we found that reducing cellular levels of ROS and blocking p38 or COX-2 activity was associated with a significant reduction ($p < 0.05$) in Cd-induced PGE_2 production at 6 hours (Fig. 10). Altogether, our data suggest that Cd increases cellular migration of colon cancer cells, through induction of ROS-mediated activation of p38 MAPK which subsequently upregulates the expression of COX-2 leading to accumulation of PGE2.

Cd activates Akt in a ROS-dependent pathway

The effect of Cd on the Akt, another signaling pathway actively involved in the migration of cancer cells was investigated. We found that Cd caused a significant increase in the level of phosphorylated Akt detectable as early as 30 minutes post-treatment with Cd ($p <0.05$ and 0.01 respectively) (Fig. 11A and B). Inhibition of ROS generation by NAC significantly attenuated Cd-induced Akt phosphorylation at 60 minutes (Fig. 11C and D).

Akt is a crucial mediator of Cd-induced migration

Next, we examined whether Cd-mediated activation of Akt signaling pathway is involved in the Cd-induced increase in colon cancer cell migration. We show that the inhibition of PI3K and thereby Akt, by the PI3K inhibitor LY294002, significantly ($p < 0.05$) abrogated Cd-increased migration. Pre-treatment with both CLX and LY294002 did not provide additional decrease in migration when compared to LY294002 or CLX alone (Fig. 12A and B).
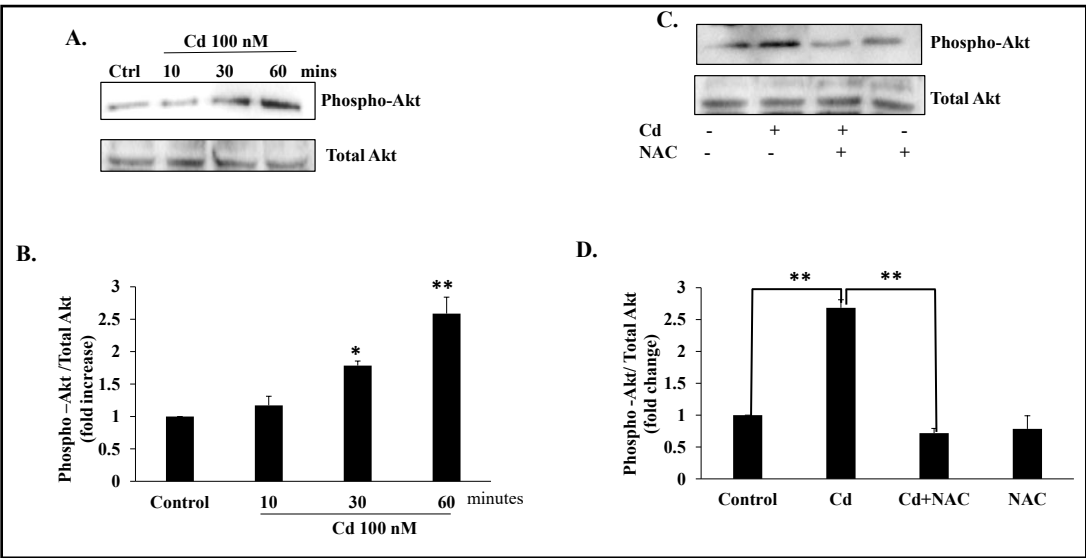
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Fig. 11. Cd activates Akt in a ROS mediated pathway. (A) and (B). HT-29 cells were treated with Cd (100 nM) for 0, 10, 30 and 60 minutes and Akt phosphorylation levels were assessed by western blotting. (C) and (D): cells were pre-treated with NAC (10 mM) for 30 minutes followed by Cd (100 nM) for 60 minutes and Akt phosphorylation levels were determined by western blotting. Values are represented as mean ± SEM of n=3. * denotes $p<0.05$, ** denotes $p<0.01$.
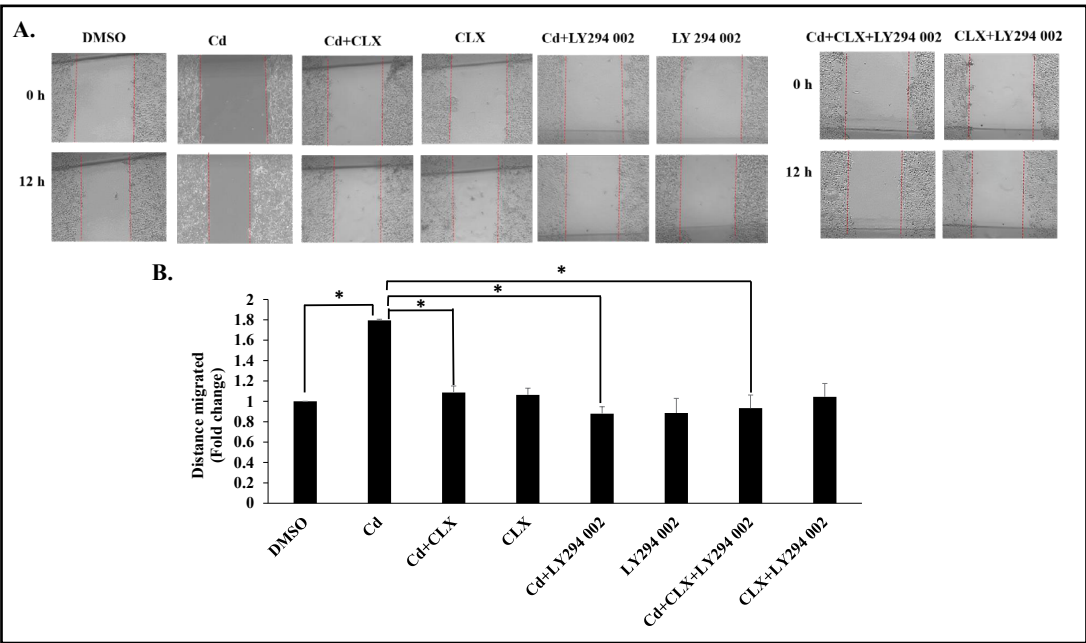

Fig. 12. Cd-induced Akt activation is implicated in migration. HT-29 cells were treated with Cd (100 nM) in the presence or absence of LY294 002 (10 μM), a PI3K inhibitor and/or CLX. Migration was evaluated using scratch wound healing assay. Values are represented as mean ± SEM of n=3.* denotes $p<0.05$.

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Discussion

Although Cd is a known human carcinogen, the underlying signaling pathways implicating it in colorectal malignancy are not well characterized. In this report, the effect of Cd on CRC cells migration was investigated. Herein, we show that Cd increases the migratory capacity of CRC cells via activating two inter-connected pathways: ROS-p38-COX-2-PGE$_2$ and the ROS-Akt pathway.

It is widely accepted that inflammation plays an important role at different stages of tumor development [25, 26]. Overwhelming evidence shows that the cyclooxygenase-mediated pathway is a primary pro-inflammatory signaling cascade implicated in colorectal malignancy [43]. In fact, expression of COX-2 was reported to be increased in human colorectal cancer mucosa when compared to the normal tissues [29]. Epidemiological evidence demonstrates that chronic aspirin use is associated with 40- 50% reduction in CRC relative risk [44]. Indeed, selective inhibition of COX-2 is associated with reduced CRC cell proliferation [45], migration and invasion [32]. Here, we report that Cd causes a significant increase in cellular migratory capacity. To investigate the underlying mechanisms of Cd-induced cell migration, we looked at COX-2 expression. Interestingly, Cd caused a time dependent increase in the transcription and protein expression of COX-2. The fact that Cd upregulated COX-2 as early as 6 hours most likely indicates a post-transcriptional regulation, which could be explained by either increased COX-2 translational efficiency or mRNA stability. To assess whether Cd-induced COX-2 upregulation is positively implicated in migration, cells were pre-treated with CLX. Indeed, blocking COX-2 activity abolished Cd-induced migration, clearly indicating that Cd, via increasing COX-2 expression, is enhancing migration. This is consistent with several lines of evidence which established that COX-2 plays a crucial role in promoting migration of cancer cells [46, 47].

It is well known that CLX and other NSAIDs reduce carcinogenesis via inhibiting COX-2 activity. Nevertheless, COX-2 independent anti-tumor effects of NSAIDs have been also reported [48]. For instance, CLX was reported to reduce membrane fluidity and hence metastatic potential of CRC cells in a COX-2 independent mechanism [49]. Conversely, COX-2 positive but not COX-2 negative CRC cells treated with CLX showed a significant reduction in Epithelial-mesenchymal transition (EMT), strongly implying a COX-2 dependent anti-tumor mechanism [50]. Moreover, it has been reported that CLX tends to accumulate in the hydrophobic interior of plasma membrane of different cell types including B lymphocytes, vascular endothelial cells, leukemic monocytes and human colorectal cancer cells, and thus a higher intracellular concentration is needed to observe its anti-carcinogenic effects [51]. Accordingly, the concentration of CLX used in the present study is 10 μM, which is higher than the maximal plasma concentration achieved (3-5 μM) following the administration of 800 mg/day CLX [52].

A huge body of evidence indicates that ROS plays an important role in different types of cancer including CRC [53]. Several signaling pathways have been implicated in ROS-mediated carcinogenicity. In fact, ROS mediates the activation of Src kinases, PI3K/Akt, and MAPKs, all of which promote malignancy [54]. Previous findings support the notion that ROS could promote EMT [55] as well as tumor cell migration and invasion [56]. It is not surprising then, that reducing cellular levels of ROS using NAC is associated with the inhibition of migration of several cancer cells [57, 58]. The present study established that Cd induces ROS production as early as 5 minutes. This is in agreement with literature which indicates that Cd is an oxidative stressor [12]. Moreover, it has been suggested that ROS plays a crucial role in Cd-induced carcinogenicity [13]. To explore whether oxidative stress is involved in Cd-induced migration, cells were pretreated with the ROS scavenger NAC. Indeed, decreasing cellular ROS levels attenuated Cd- increased cell migration. Interestingly, ROS was reported to upregulate COX-2 in CRC [53]. In line with this, our results show that reducing oxidative stress led to a significant decrease in Cd-induced COX-2 expression, clearly implicating ROS as a COX-2 positive modulator.

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It is important to note that in addition to its tumor promoting role, a tumor suppressive role of ROS has been also reported [59]. Contrary to our findings, Piskounova et al., demonstrated that NAC promoted metastasis in melanoma human xenografts [60]. Similarly, NAC markedly increased migration and invasion of human malignant melanoma cells [61]. Moreover, dietary supplementation with antioxidants such as NAC or Vitamin E was shown to accelerate the progression of lung cancer in mice [62]. It is now accepted that low levels of ROS support cells proliferation, migration and survival whereas high levels are generally cytotoxic [53, 63].

Aberrant MAPK signaling plays a crucial role in the development and progression of different malignancies [64]. Three main groups of MAPKs have been identified in mammals: ERK1/2, JNK 1/2/3 and p38 MAPK [65]. The MAPKs are activated by variety of stimuli such as growth factors, pro-inflammatory cytokines, hormones and oxidative stress [66]. Indeed, being an oxidative stressor, Cd was reported to activate MAPKs [67]. Contextually, herein we report that Cd significantly increases p38 MAPK phosphorylation in a ROS mediated pathway. Previous studies indicate that p38 is implicated in several human diseases including cancer [68]. In fact, elevated levels of phosphorylated p38 have been associated with various malignancies [69]. A recently published study shows that p38 MAPK is involved in the migration of human breast cancer cells [70]. A positive association has been reported between high endogenous p38 MAPK activity and cancer cells invasiveness [71]. Additionally, pro-proliferative [72] and anti-apoptotic effects were correlated with p38 activation [73, 74]. The fact that SB202190 abolished Cd-induced HT-29 migration is consistent with reports implicating active p38 as a mediator of cancer cells migration [72]. Moreover, we have shown that Cd-induced increase in COX-2 transcriptional activity and protein expression was significantly attenuated by SB202190. This is in agreement to previous findings suggesting that the activation of p38 could contribute to cancer progression via upregulating COX-2 in different tumors [69]. It is noteworthy that besides its tumor promoting effect, a tumor suppressive role of p38 has been also described [75]. In fact, increasing evidence highlights the pro-apoptotic roles of p38 [75]. Remarkably, p38 activity appears to be essential for some chemotherapeutic drugs induced apoptosis. The alkylating agent Cisplatin for example, was shown to induce a p38 mediated tumor cell death [76]. Importantly, the function of p38 MAPK in cancer appears to depend on the cell type, the stimuli and/or the isoform that is activated [74].

Because PGE_2 is thought to be the major pro-tumorigenic metabolite of COX-2, we next explored its effect on HT-29 migration. Our findings indicate that the addition of PGE_2 significantly potentiated migration. These results support the notion that PGE_2 is an important mediator of colon cancer cells migration [39, 40]. Previous studies yielded conflicting results regarding the catalytic activity of COX-2 in HT-29 cell line. Parker et al. demonstrated that the addition of arachidonic acid (AA), a COX substrate, simulates the production of PGE_2 in HT-29 [77]. On the contrary, other investigators have shown that although COX-2 is expressed in HT-29 cells, it is catalytically inactive and is therefore unable to convert AA to prostaglandins [78]. Our results indicate that Cd causes a significant rise in PGE_2 production, clearly suggesting that the Cd-induced de novo synthesized COX-2 is enzymatically active. The present study further established Cd-induced PGE_2 production was significantly attenuated by NAC, SB202190 and CLX pre-treatment. These findings support the fact that PGE_2 is major COX-2 product and that ROS and p38 are critical mediators of COX-2 expression.

Substantial evidence indicates that the PI3K/Akt pathway is implicated in cancer [79]. In fact, this pathway plays an important role in promoting cell proliferation, survival, invasion, metastasis and angiogenesis [79]. The selective PI3K inhibitor LY294002 was shown to significantly decrease ovarian cancer cells proliferation [80], invasion and metastasis [81]. As a matter of fact, aberrant PI3K/Akt pathway occurs in several malignancies including CRC [79]. Interestingly, when compared to normal colonic mucosa, colorectal adenomas and carcinomas frequently overexpress Akt, the major downstream player of PI3K [82]. Importantly, Turečková et al. established that Akt strongly stimulates the migration of colorectal

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cancer cells via phosphorylating focal adhesion kinase (FAK) in a Src mediated pathway [83]. In line with this, our results indicate that Cd significantly increases the phosphorylation of Akt. Furthermore, inhibiting Akt significantly abolished Cd-increased migration, clearly implicating Akt as a mediator of migration. Our findings further demonstrate that inhibiting both COX-2 and Akt was not associated with any additional decrease in migration. The exact explanation remains to be fully elucidated; however, the following could be postulated: Cd-induced PGE_2 production before 1 hour could actually be activating the PI3K/Akt pathway. Previous reports indicated that PI3K/Akt pathway is an important downstream mediator of the COX-2/PGE_2 pathway. In fact, PGE_2 was shown to enhance the growth, migration and invasion of colorectal cancer cells in PI3K/Akt dependent pathway [41]. Since the PI3K/Akt pathway is known to be activated by ROS [84], we determined if indeed Cd-induced Akt phosphorylation is ROS-mediated. Herein, we showed that pre-treatment with NAC significantly attenuated Cd-induced Akt phosphorylation, strongly suggesting that ROS is upstream of Akt.

Increasing evidence highlights the role of EP receptors in colorectal neoplasia. Elevated EP4 receptor expression was reported in colorectal cancers (100%) as well as adenomas (36%) when compared with normal colon epithelium [36]. Enhanced tumorigenic behavior and suppression of apoptosis was observed in HT-29 human adenocarcinoma cells overexpressing EP4 receptors [85]. Moreover, the genetic deletion or the pharmacological inhibition of EP4 receptor [86] and EP1 receptor [87] caused a significant inhibition of tumor growth, suggesting that these receptors play a key role in colon tumorigenesis.

Conclusion

The findings of the present study argue that exposure to low Cd levels enhances migration of CRC cells via induction of the ROS-p38-COX-2-PGE_2 and the ROS-Akt pathway. Therefore, COX-2, PGE_2 receptors, or Akt may represent potential targets in the treatment of CRC, specifically in patients exposed to Cd.

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Disclosure Statement

The authors declare that they have no competing interests.

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