Published OnlineFirst July 7, 2009; DOI: 10.1158/1541-7786.MCR-08-0493

Cyclooxygenase-2 Up-Regulates Ataxia Telangiectasia and Rad3 Related through Extracellular Signal-Regulated Kinase Activation

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Abstract

Cyclooxygenase-2 (COX-2) overexpression caused prolonged G_2 arrest after exposure to ionizing radiation (IR) in our previous study. We were therefore interested in investigating the function of COX-2 in the G_2 checkpoint pathway. Interestingly, we found that cells in which COX-2 is overexpressed showed up-regulated ataxia telangiectasia and Rad3 related (ATR) expression compared with control cells. In this study, we investigated the mechanism of ATR up-regulation by COX-2 and tested our hypothesis that COX-2–induced extracellular signal-regulated kinase (ERK) activation mediates up-regulation of ATR by COX-2. To investigate the relationship between COX-2 and ATR, we used two stable COX-2–overexpressing cancer cell lines (HCT116–COX-2 and H460–COX-2), a COX-2 knockdown A549 lung cancer cell line (AS), and an ATR knockdown HCT116 cell line. Cells were treated with various drugs [celecoxib, prostaglandin E_2 (PGE_2), PD98059, U0126, and hydroxyurea] and were then analyzed using reverse transcription-PCR, confocal microscopy, Western blotting, and clonogenic assay. COX-2–overexpressing cells were shown to have increased ERK phosphorylation and ATR expression compared with control cells, whereas AS cells were shown to have decreased levels of phospho-ERK and ATR. In addition, exogenously administered PGE_2 increased ERK phosphorylation. Inhibition of ERK phosphorylation decreased ATR expression in both HCT116–COX-2 and A549 cells. HCT116–COX-2 cells were resistant to IR or hydroxyurea compared with HCT116-Mock cells, whereas administration of ATR shRNA showed the opposite effect. COX-2 stimulates ERK phosphorylation via PGE_2. This COX-2–induced ERK activation seems to increase ATR expression and activity in endogenous COX-2–overexpressing cancer cells as well as in COX-2–overexpressing stable cell lines. (Mol Cancer Res 2009;7(7):1158–68)

Received 10/20/08; revised 4/14/09; accepted 4/16/09; published OnlineFirst 7/7/09.
Grant support: National Cancer Center grant 0710380-1.
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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).
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doi:10.1158/1541-7786.MCR-08-0493

Introduction

Monitoring heritable genetic errors is critical for maintaining genomic integrity (1-3). Organisms can be exposed to many environmental genotoxic stresses, such as UV light, ionizing radiation (IR), and reactive chemicals, which can cause damage to the genome (4). All eukaryotes have developed devices that can detect DNA damage and transmit signals to downstream effectors to repair the damage or trigger apoptosis in cells with damaged DNA (2, 3). A number of cancer cells have one or more defects in DNA damage response pathways, and this fact indicates that DNA damage response pathways play important roles in limiting cancer development (1). In eukaryotes, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are essential for many DNA damage responses (1-3, 5). ATM and ATR belong to phosphoinositol 3-kinase–related kinases. They are cell cycle checkpoint kinases; in their active form, they inhibit the transition of cells with damaged DNA from G_2 into the mitotic (M) phase. They have sequential/structural homology and share the same substrates for specific DNA damage repair pathways (6-9). Although ATM and ATR cross talk, they respond differently to genotoxic stresses. When DNA is damaged, the ATM/checkpoint kinase 2 (Chk2) pathway is activated at an early time point, whereas activation of the ATR/Chk1 pathway occurs at a later stage (10, 11). ATM/Chk2 responds primarily to DNA double strand breaks, whereas ATR/Chk1 is activated by DNA replication fork stalling and bulky DNA lesions (2, 3, 5, 6, 11). The deletion of the *ATR* gene causes early embryonic lethality in mice, and the mutation of this gene results in Seckel syndrome, which is characterized by a defective DNA damage response (10-12). Loss of ATR function also inhibits phosphorylation of downstream effectors such as p53 and Chk1 and leads to apoptosis (6, 13). These facts indicate that ATR is essential for cell survival, development, and DNA damage response pathways (10, 11).

Cyclooxygenase (COX) is an enzyme that catalyzes the conversion of arachidonic acid to prostaglandins. COXs have two isoenzymes, COX-1 and COX-2, and are located in the endoplasmic reticulum (ER) and inner/outer nuclear envelope membrane. COX-1 is constitutively expressed and detected in most human tissues. In contrast, COX-2 is transiently induced by various stimuli and is involved in complex carcinogenic processes, such as transformation, angiogenesis, inflammation, invasion, and metastasis, by generating prostaglandin E_2 (PGE_2) or by inducing the expression of various angiogenic factors (14, 15). In addition, PGE_2 stimulates cell proliferation through the activation of extracellular signal-regulated kinase (ERK), which can result in cancer development (16-19). For these reasons,

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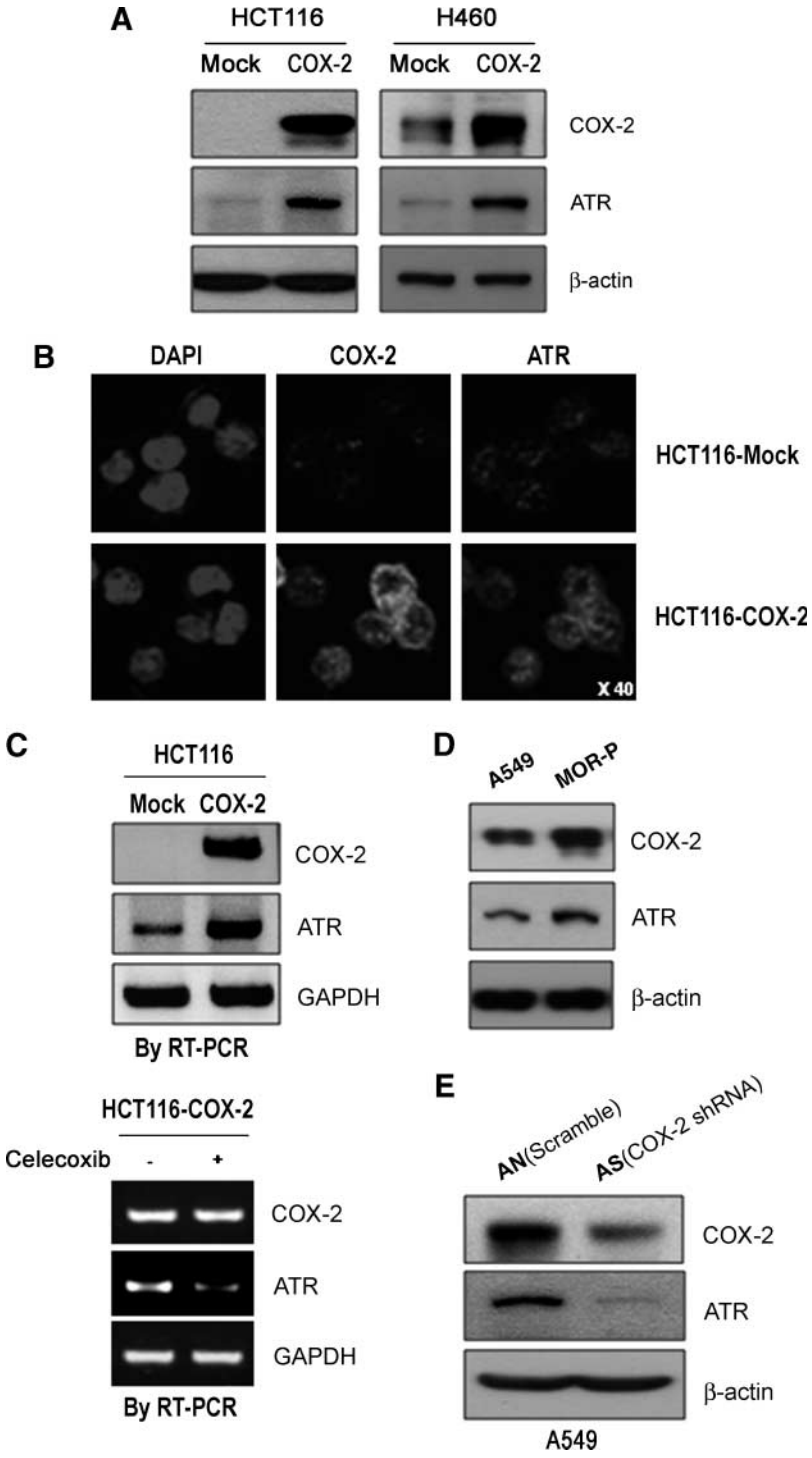

FIGURE 1. COX-2 up-regulates expression of ATR in colon cancer cells (HCT116) and lung cancer cells (H460, A549, and MOR-P). **A.** Expression levels of COX-2 and ATR were analyzed using Western blotting in HCT116-Mock/HCT116–COX-2 cells and H460-Mock/H460–COX-2 cells. **B.** HCT116-Mock and HCT116–COX-2 cells were stained with ATR and COX-2–specific antibodies, and the images were taken with confocal microscopy as described Materials and Methods. **C.** Transcription levels of COX-2 and ATR were analyzed using RT-PCR in normal HCT116-Mock and HCT116–COX-2 cells (*top*) or HCT116–COX-2 cells treated without or with 50 μmol/L celecoxib for 4 h (*bottom*). COX-2, ATR, and β-actin were analyzed by Western blotting in A549 and MOR-P (**D**), and AN (control) and AS (COX-2 knockdown A549 cells) stable cells (**E**). All measurements were done at least in triplicate.

COX-2–specific inhibitors, including celecoxib, have been used in cancer cells to inhibit PGE_2 generation and thereby to suppress their proliferation (20). Celecoxib can also induce cell cycle arrest, apoptosis, or ERK activation through a COX-2–independent mode (21, 22). These facts indicate that celecoxib acts through COX-2–dependent and COX-2–independent pathways (23-26).

ERK is associated with various cellular complex processes such as cell survival, proliferation, gene expression, and migration (16, 18, 27). Several studies have suggested that DNA-damaging stimuli such as UV light, hydroxyurea, and IR induce ERK activation and result in G_2-M arrest (23, 27, 28). In addition, when cells are exposed to DNA damage–inducing agents, ERK inhibition decreases ATR activity and results in

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decreasing Chk1 activation and repression of G_2-M arrest (23). Taken together, these facts suggest that ERK activation may play a key role in the regulation of the ATR/Chk1–dependent G_2-M checkpoint in cells with damaged DNA.

In our previous study, we found that COX-2 overexpression caused prolonged G_2 arrest after exposure to IR and suggested that COX-2 may contribute to the regulation of the G_2 checkpoint (29). Therefore, we have been interested in discovering and characterizing target molecules of COX-2 in the G_2 checkpoint pathway. Interestingly, among several molecules differentially regulated by COX-2, we found that COX-2–overexpressing cells always have up-regulated ATR expression compared with controls. Therefore, we investigated how COX-2 up-regulates ATR expression in the absence of DNA damage and whether the elevated ATR can protect cells from DNA-damaging stimuli.

In this study, we report that overexpressed COX-2 activates ERK via PGE_2 and the activated ERK, not DNA damage response, enhances ATR kinase expression and activity in COX-2–overexpressing cancer cells.

Results

Up-Regulation of ATR Expression by COX-2

We developed two stable COX-2–overexpressing cancer cell lines (HCT116–COX-2 and H460–COX-2) and a COX-2 knockdown A549 human lung adenocarcinoma cell line (AS) as described in Materials and Methods. Among the molecules that are involved in the G_2 checkpoint, we searched the ones differentially regulated by COX-2 using HCT116-Mock versus HCT116–COX-2 cells, and AN versus AS cells.

Among tested molecules involved in the G_2 checkpoint,[1] we found that HCT116–COX-2 and H460–COX-2 cells showed up-regulated ATR expression compared with control cells (HCT116-Mock and H460-Mock) using Western blot analysis and confocal microscopy (Fig. 1A and B). To understand the mechanism of COX-2–induced ATR overexpression, we performed reverse transcription-PCR (RT-PCR) using total RNA extracted from untreated or celecoxib-treated HCT116-Mock and HCT116–COX-2 cells. We found that COX-2 increased ATR transcription but celecoxib inhibited it (Fig. 1C). To determine the COX-2 dependency of ATR expression in other cells, we investigated whether COX-2 regulates ATR expression in A549 and MOR-P lung cancer cells. Both A549 and MOR-P cells show high expression levels of endogenous COX-2, and the levels of COX-2 and ATR expression in MOR-P were higher than those in A549 cells (Fig. 1D). In addition, AS cells showed significantly decreased ATR expression compared with AN control cells (Fig. 1E). These results suggest that COX-2 may be involved in regulation of ATR expression.

Next, we examined whether ATR up-regulation in HCT116–COX-2 cells is the result of a transcriptional activation or an increase in ATR mRNA stability. HCT116-Mock and HCT116–COX-2 cells were treated with actinomycin D for the indicated times (0, 2, and 4 hours) to inhibit transcription and then the degradation rate of ATR mRNA was analyzed by RT-PCR. The degradation rate of ATR mRNA in HCT116–COX-2 cells was similar to HCT116-Mock cells (Supplementary Fig. S1). This result indicates that COX-2–induced ATR up-regulation is not caused by an increase of mRNA stability. In conclusion, COX-2 seems to increase ATR expression by activating ATR transcription.

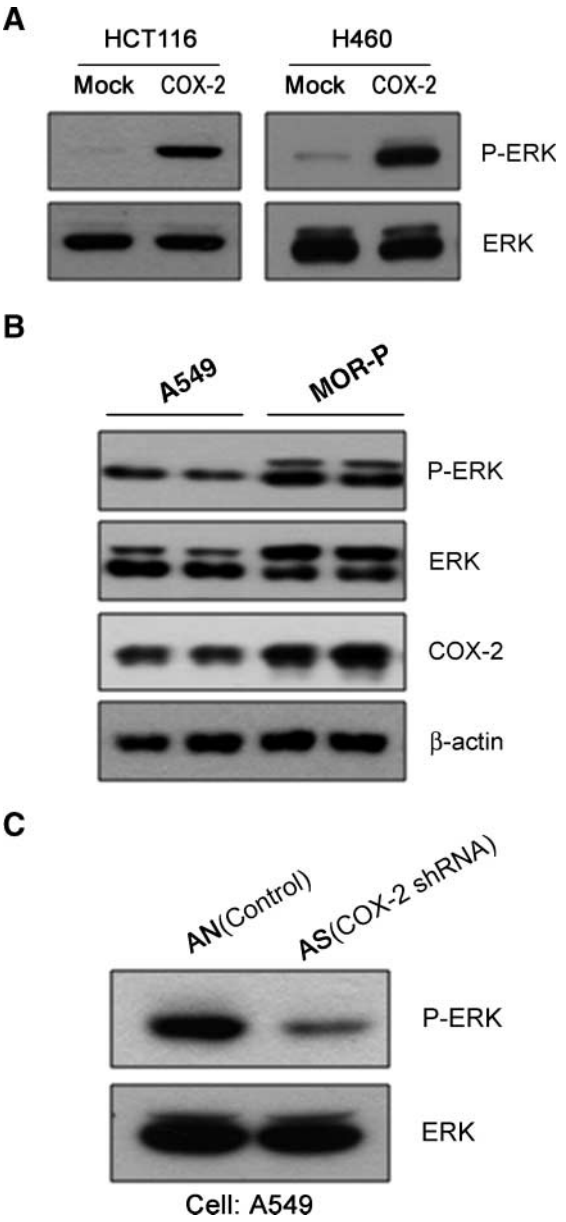

FIGURE 2. Overexpressed COX-2 induces activation of ERK in HCT116 colon cancer cells as well as lung cancer cells (H460, A549, and MOR-P). **A.** ERK phosphorylation of untreated Mock and COX-2 stable cells (HCT116 and H460) was detected by phospho-ERK and ERK-specific antibody. Phospho-ERK and ERK were analyzed by Western blotting in A549 and MOR-P (**B**), and AN (control) and AS (COX-2 knockdown A549 cells) stable cells (**C**). All measurements were done at least in triplicate.

COX-2 Induces ERK Activation

Recently, several articles have reported that activated ERK regulates ATR activity (27, 28, 30). Although these findings are

[1] Unpublished data.

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on the ERK regulation of ATR activity and not on ATR expression, we examined whether ERK is involved in COX-2–induced ATR expression. Interestingly, HCT116–COX-2 and H460–COX-2 cells showed increased ERK phosphorylation compared with their control cells (Fig. 2A). ERK phosphorylation increased proportionally to COX-2 expression in A549 and MOR-P cells (Fig. 2B). In contrast, AS cells showed decreased ERK phosphorylation compared with AN cells (Fig. 2C). These results suggest that COX-2 may regulate ERK activity at the upstream position and propose the possibility that COX-2 may induce ERK activation and result in increased ATR expression.

COX-2 Regulates ERK Activation via PGE_2

Next, we studied how COX-2 induces ERK activation. To investigate whether PGE_2, a major end product of COX-2, is associated with ERK activation, cells were treated with 500 nmol/L PGE_2. A concentration of 500 nmol/L PGE_2 is equivalent to the maximum inducible concentration in A549 cells, which have higher expression levels of COX-2 compared with those of HCT116 cells, and this amount, although sufficient to exert a considerable effect, does not exceed its physiologic range (29).

HCT116-Mock and HCT116–COX-2 cells were treated with PGE_2 for the indicated times (0, 4, 8, and 24 hours), and then ERK phosphorylation and ATR expression were analyzed. PGE_2 treatment increased ERK phosphorylation in HCT116-Mock cells, although the level of phosphorylated ERK achieved in HCT116-Mock cells after PGE_2 treatment did not reach that of HCT116–COX-2 cells without PGE_2 treatment (Fig. 3A). ATR expression was increased by PGE_2 administration in both HCT116-Mock and HCT116–COX-2 cells. The amount of ATR increase in HCT116-Mock cells at 24 hours after PGE_2 treatment was equivalent to that of HCT116–COX-2 cells at 0 h without PGE_2 treatment (Fig. 3A). These results suggest that PGE_2 may be responsible for ERK activation and increased ATR expression in COX-2–overexpressing HCT116 cells.

When PGE_2 was administered in A549 and MOR-P cells, which are cell lines that constitutively express high levels of COX-2, the pattern of ERK activation was similar to that seen in HCT116 cells. PGE_2 also increased ERK phosphorylation in both cell lines and the level of ATR expression followed the pattern of ERK phosphorylation at earlier time points (4 and 8 hours), although ATR expression did not decline in MOR-P cells at 24 hours when ERK phosphorylation decreased (Fig. 3B). Taken together, COX-2 seems to induce ERK activation via PGE_2 in COX-2–overexpressing cells.

We further investigated whether PGE_2 increases ATR transcription. After HCT116-Mock and A549 cells were treated with PGE_2, ATR mRNA levels were analyzed by RT-PCR. Figure 3C shows that PGE_2 increased ATR transcription in both tested cell lines.

Based on these results, we suggest that COX-2–induced PGE_2 stimulates ERK phosphorylation and activated ERK governs ATR expression in COX-2–overexpressing cancer cell lines.

COX-2–Induced ERK Activation Regulates ATR Expression

To confirm whether COX-2–induced ERK activation up-regulates ATR expression, HCT116-Mock and HCT116–COX-2 cells were treated with PD98059 at various concentrations (0, 25, 50, and 100 μmol/L) for 8 hours at 37°C. ERK phosphorylation was significantly inhibited by 100 μmol/L PD98059 in these cells (Fig. 4A). Based on this result, cells were subsequently treated with 100 μmol/L PD98059 for the indicated times (0, 4, 8, and 24 hours) at 37°C. ERK phosphorylation

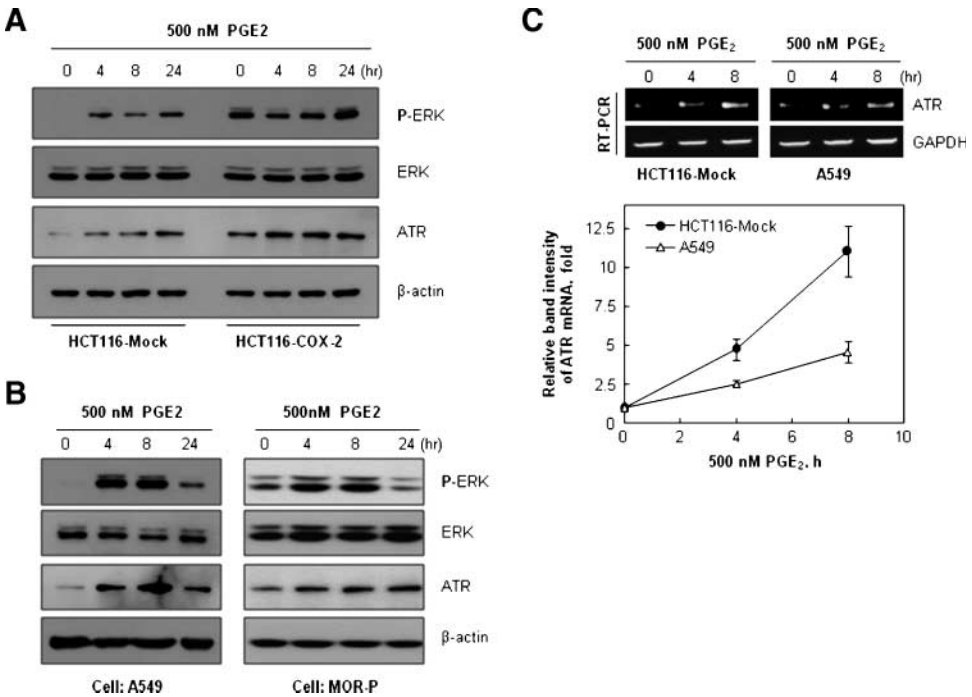

FIGURE 3. PGE_2 activates ERK and results in ATR increase in HCT116 colon cancer cells and lung cancer cells (A549 and MOR-P). Cells were incubated with 500 nmol/L PGE_2 for the indicated times (0, 4, 8, and 24 h) at 37°C and then phospho-ERK, ERK, ATR, and β-actin were detected by specific antibodies. HCT116-Mock and HCT116–COX-2 cells (**A**), A549 and MOR-P cells (**B**), and HCT116-Mock and A549 cells (**C**) were treated with 500 nmol/L PGE_2 for the indicated times (0, 4, and 8 h) and then levels of ATR mRNA were measured by RT-PCR and quantified as described in Materials and Methods. All measurements were done at least in triplicate. 0 h, 0.1% DMSO control.

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was inhibited from 4 hours after PD98059 treatment and completely inhibited after 24 hours in HCT116–COX-2 (Fig. 4B) and A549 cells (Fig. 4C); however, ATR expression was inhibited after 24 hours of treatment in both cell lines. Although 100 μmol/L of PD98059 is a relatively high concentration compared with that used in other studies, this concentration was not toxic to either the HCT116 or A549 cells in our experimental system (data not shown). To further confirm that ERK regulates ATR expression, HCT116–COX-2 and A549 cells were treated with another ERK inhibitor, U0126. U0126 completely inhibited ERK phosphorylation within 30 minutes after treatment, but inhibition of ATR expression was shown after 4 hours of treatment in HCT116–COX-2 cells (Fig. 5A, *left*) and A549 cells (Fig. 5A, *right*). This time sequence of inhibition of ERK phosphorylation and ATR expression suggests that ERK inhibition in COX-2–overexpressing cells down-regulates ATR expression.

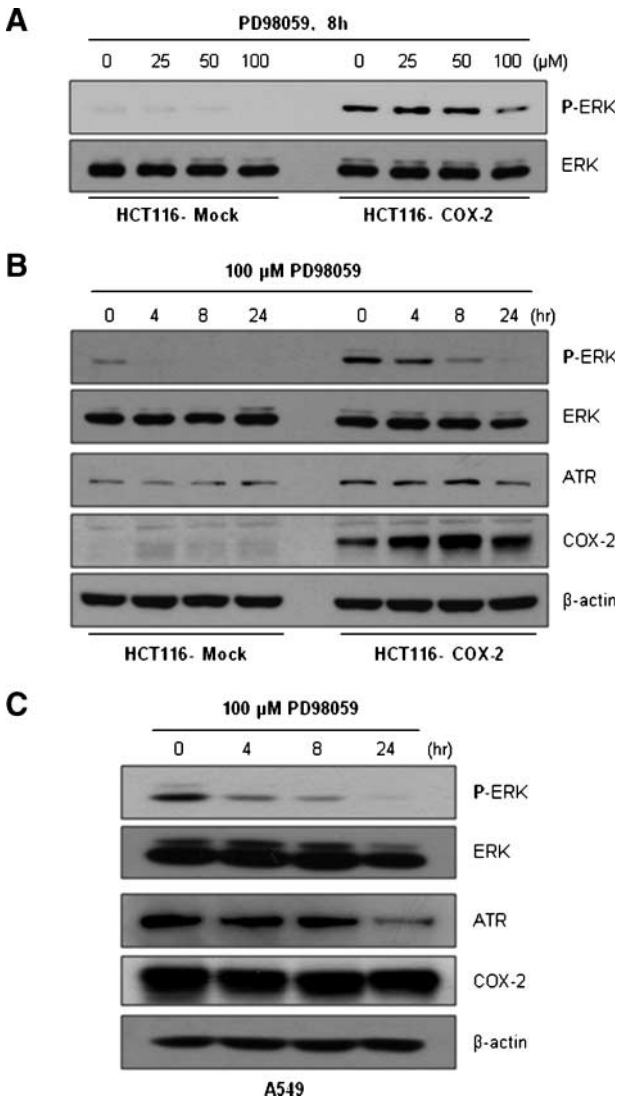

FIGURE 4. Inhibition of phospho-ERK induces down-regulation of ATR expression in both HCT116 colon cancer cells and A549 lung cancer cells. HCT116-Mock and HCT116–COX-2 cells and A549 cells were exposed to a vehicle (DMSO 0.1%) or to ERK inhibitors at 37°C as follows: PD98059 at indicated concentrations (0, 25, 50, 100 μmol/L) for 8 h (**A**) and 100 μmol/L PD98059 for the indicated times (0, 4, 8, and 24 h; **B** and **C**).

Next, to further confirm that activated ERK regulates ATR transcription, HCT116–COX-2 and A549 cells were treated with PD98059 for 24 hours, and then ATR mRNA levels were measured by RT-PCR. Figure 5B shows that PD98059 completely inhibited ATR transcription in both HCT116–COX-2 cells and A549 cells. Therefore, these results further suggest that COX-2–induced ERK activation may be responsible for up-regulated ATR expression in COX-2–overexpressing cells.

HCT116–COX-2 Cells Show Elevated Chk1 and p53 Phosphorylation Dependent on ERK Activity

Currently, it is not well known whether increased ATR expression is correlated with increased ATR activity. ATR is activated by DNA damage and it initiates downstream signaling cascades through phosphorylation of its direct target molecules, including Chk1 and p53 (2, 3, 31).

First, we examined whether HCT116–COX-2 cells induce phosphorylation of Chk1 under basal condition. HCT116–COX-2 cells showed increased phosphorylation of Chk1 compared with HCT116-Mock cells (Fig. 6A). Next, we examined whether ERK inhibition by PD98059 had an effect on the phosphorylation of downstream effectors of ATR, Chk1, or p53. When HCT116-Mock and HCT116–COX-2 cells were treated with 100 μmol/L PD98059 for the indicated times (0, 4, 8, and 24 hours), Chk1 and p53 phosphorylation decreased to levels lower than those of the control after 24 hours of treatment (Fig. 6A). These results indicate that COX-2–induced ERK activation may positively regulate ATR activity as well as expression, subsequently inducing phosphorylation of Chk1 and p53.

COX-2 Does Not Induce DNA Damage Response

Next, we examined whether HCT116–COX-2 cells induce DNA damage response under basal condition. When HCT116–COX-2 cells were exposed to 6 Gy IR, γ-H2AX nuclear foci were clearly seen; however, we could not detect γ-H2AX nuclear foci in unirradiated HCT116-Mock and HCT116–COX-2 cells (Fig. 6B). We also could not detect nuclear foci of ATR in HCT116–COX-2 cells under basal condition (data not shown). These results suggest that elevated ATR in COX-2–overexpressing cells is not a result of DNA damage response induction by COX-2.

HCT116–COX-2 Cells Containing Elevated ATR Are Resistant to DNA-Damaging Stimuli

To verify whether cells in which ATR is up-regulated by COX-2 are resistant to DNA-damaging stimuli, cells were exposed to DNA-damaging agents such as IR or hydroxyurea. IR is a well-known inducer of DNA double strand break. Hydroxyurea, a competitive inhibitor of ribonucleotide reductase, blocks DNA replication, resulting in ATR activation (13, 32). HCT116-Mock and HCT116–COX-2 cells were exposed to graded doses of IR or hydroxyurea, and then cell viability was monitored by a clonogenic assay. HCT116–COX-2 cells showed significant resistance to IR and hydroxyurea compared with control cells (Fig. 7A).

Next, we developed ATR knocked down stable cell line using ATR shRNA and examined whether ATR is related with radioresistance in HCT116–COX-2 cells. HCT116-SC and

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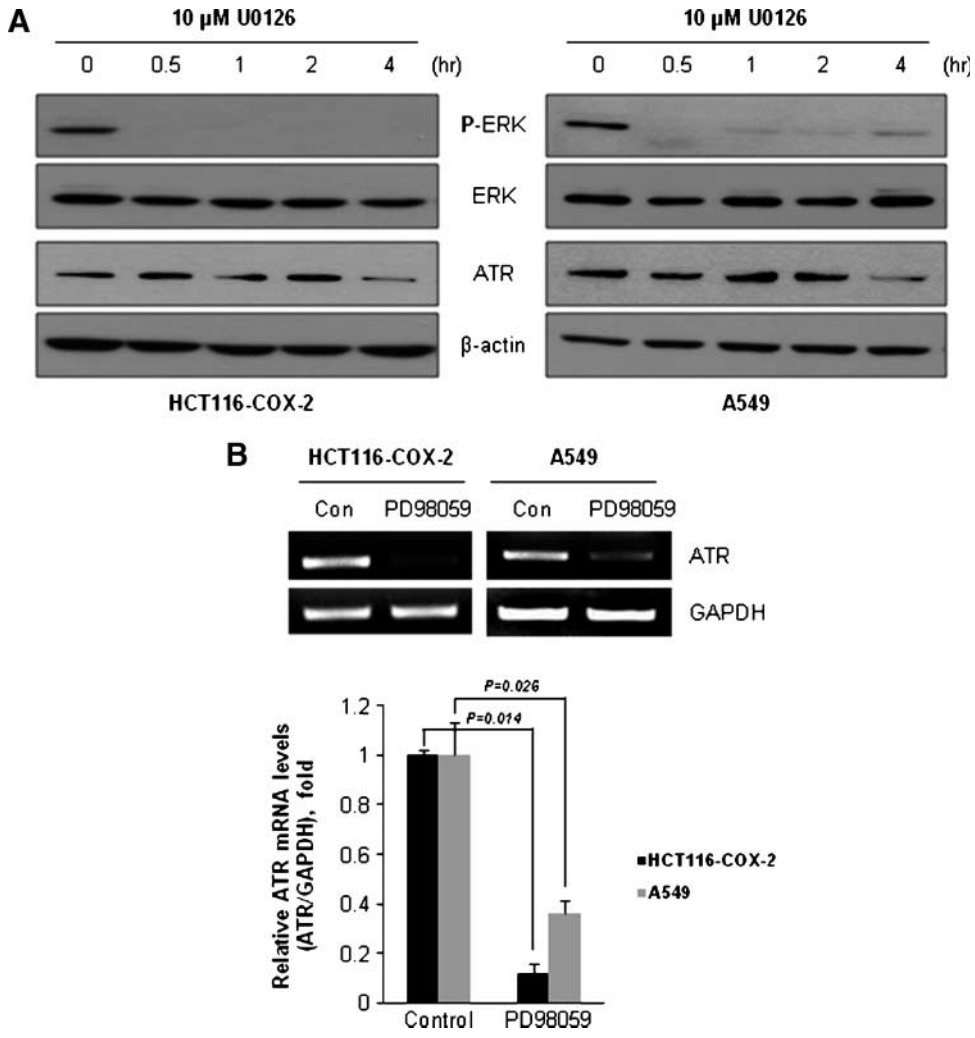

FIGURE 5. **A.** U0126 (10 μmol/L) treatment for the indicated times (0, 0.5, 1, 2, and 4 h). Treated cells were harvested and an equal amount of protein was loaded in SDS-PAGE and detected by phospho-ERK, ERK, ATR, COX-2, and β-actin–specific antibody. **B.** HCT116-Mock and A549 cells were treated with 100 μmol/L PD98059 for 24 h and then ATR mRNA levels were measured by RT-PCR and quantified as described in Materials and Methods. All measurements were done at least in triplicate.

HCT116-ATR shRNA cells were irradiated with graded doses of IR, and then cell viability was monitored. HCT116-ATR shRNA cells were shown to almost completely knock down ATR expression and were shown to have enhanced radiosensitivity compared with HCT116-SC cells (Fig. 7B, *top*).

Finally, to verify whether cells in which COX-2–induced ATR is knocked down by ATR shRNA recover sensitivity to DNA-damaging stimuli, HCT116–COX-2 cells were transfected with ATR shRNA. HCT116–COX-2–SC and HCT116–ATR shRNA cells were exposed to graded doses of IR or hydroxyurea and then cell viability was monitored by a clonogenic assay. HCT116–COX-2–ATR shRNA cells showed ~50% decreased ATR expressions and were significantly sensitive to IR and hydroxyurea compared with COX-2–overexpressing control cells (Fig. 7B, *bottom*). These results suggest that COX-2 may induce resistance to IR or other DNA-damaging agents through ATR induction.

Discussion

Interestingly, colon cancer cells (HCT116–COX-2) that overexpressed COX-2 showed up-regulated ATR kinase expression compared with their controls (Fig. 1). We also found that COX-2–overexpressing lung cancer cells (H460–COX-2) showed up-regulated ATR expression. In contrast, COX-2 knockdown cells (AS) showed suppressed ATR kinase expression. These observations suggest that COX-2 positively regulates ATR expression and that this may be a general phenomenon in most cancer cells because similar results were shown in two different types of COX-2–overexpressing cancer cells and a COX-2 knockdown cancer cell line.

Activated ATR does not seem to be modified by phosphorylation, other covalent modifications, or oligomerization (2, 3, 13, 31). Therefore, it has been difficult to detect increase in ATR kinase activity under genotoxic stress conditions. For this reason, ATR kinase activity has been measured by phosphorylation of its substrates, including Chk1 and p53 (2, 3). Interestingly, we found that COX-2–overexpressing cells have increased phosphorylation of Chk1 and p53 under basal condition, and we therefore concluded that COX-2 also increases ATR activity as well as expression.

Chk1 is a serine/threonine kinase and is a direct target molecule of ATR kinase (5, 33). ATR kinase phosphorylates Chk1 at Ser^{317} and Ser^{345} (4, 34). Chk1 regulates the timing

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and fidelity of cell cycle transition by regulating the cdc 25A protein phosphatase. Similar to ATR, loss of Chk1 function results not only in mouse embryonic lethality at the developmental early stage but also causes cells to bypass the DNA damage and DNA replication checkpoints (33, 35). p53 is also very well known to be important to maintain cell cycle fidelity, and ATR phosphorylates p53 at Ser^{15} and Ser^{37} (32, 36). These data indicate that ATR, Chk1, and p53 are crucial for maintaining genomic integrity via regulation of the cell cycle in normal cells and in cells with damaged DNA. Therefore, our observations that COX-2 enhances the activity of ATR and its substrates, including Chk1 and p53, may be important to understand the various roles of COX-2 in cells. In addition, it may serve as an underlying mechanism for our previous observations that COX-2–overexpressing cells showed prolonged IR-induced G_2 arrest (29). Prolongation of G_2 arrest after IR has been reported in AT cells and is known as "G_2 accumulation" (37). This phenomenon has been reported to occur due to overactivated ATR/Chk1 pathway in irradiated AT cells (6). Therefore, prolongation of IR-induced G_2 arrest in COX-2–overexpressing cells may be due to up-regulated ATR by COX-2. However, further study is needed to confirm whether ATR induces prolongation of IR-induced G_2 arrest.

We cannot rule out the possibility that overexpressed COX-2 may up-regulate ATR expression by causing a DNA-damaging condition. Soutoglou and Misteli also reported that prolonged binding of DNA repair factors to chromatin can elicit the DNA damage response in the absence of DNA damage (38). However, we found that unirradiated HCT116–COX-2 cells did not show γ-H2AX nuclear foci as well as control cells. In addition, COX-2–overexpressing cells showed higher basal levels of phosphorylated ERK compared with their COX-2 low-expressing counterpart, and this ATR overexpression and elevated phosphorylation of Chk1 and p53 were suppressed when cells were treated with inhibitors for ERK. These results suggest that COX-2–induced ATR up-regulation may be mediated by the ERK pathway and not by the DNA damage response. Our results are supported by several articles that have shown that mitogen-activated protein kinases, including ERK, p38, and c-Jun-NH_2-kinase, play important roles in the

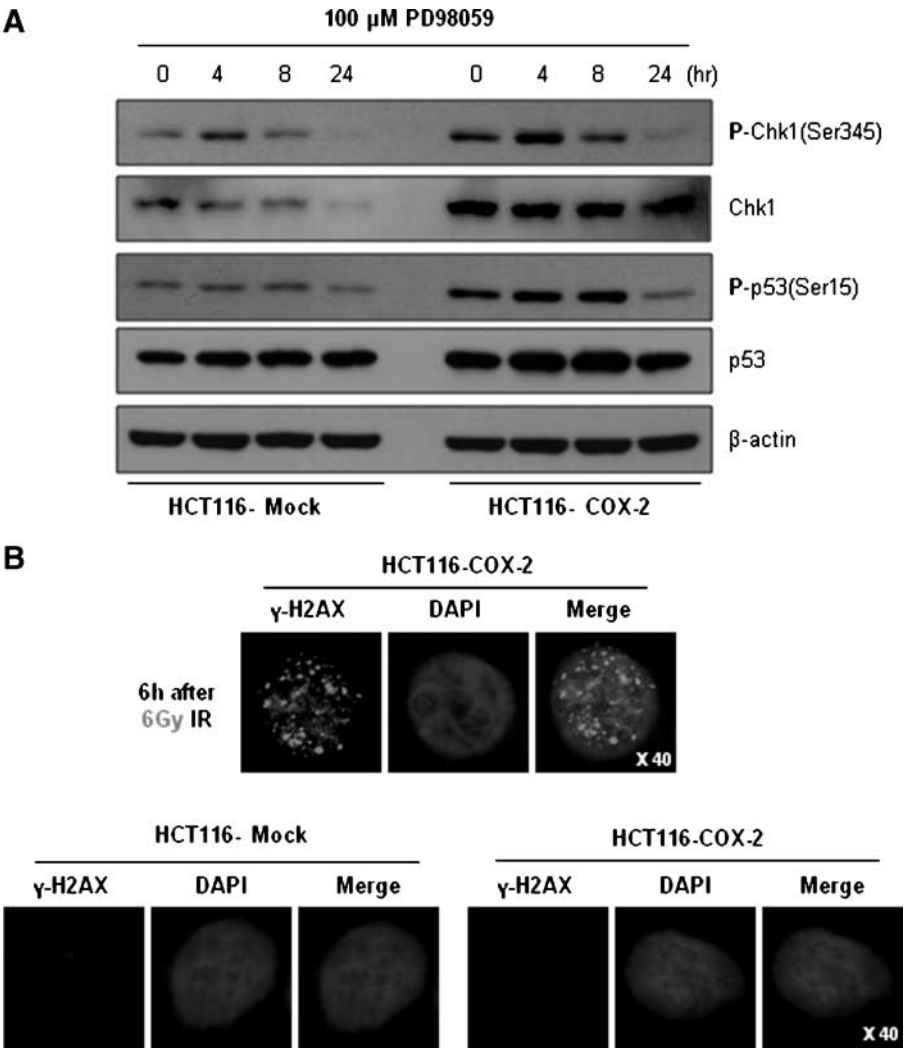

FIGURE 6. Inhibition of phospho-ERK also induces down-regulation of ATR activity in HCT116 colon cancer cells. **A.** HCT116-Mock and HCT116–COX-2 cells were exposed to a vehicle (DMSO 0.1%) or to 100 μmol/L PD98059 for the indicated times (0, 4, 8, and 24 h) at 37°C. An equal amount of protein was loaded in SDS-PAGE and detected by phospho-Chk1 (Ser^{345})/Chk1, phospho-p53 (Ser^{15})/p53, and β-actin–specific antibody. **B.** HCT116–COX-2 cells were exposed to 6-Gy IR and were then stained with γ-H2AX–specific antibodies (*top*). Undamaged HCT116-Mock and HCT116–COX-2 cells were stained with γ-H2AX–specific antibodies and followed by Alexa 594 secondary anti-rabbit IgG staining (*bottom*). The images were taken with confocal microscopy as described in Materials and Methods.

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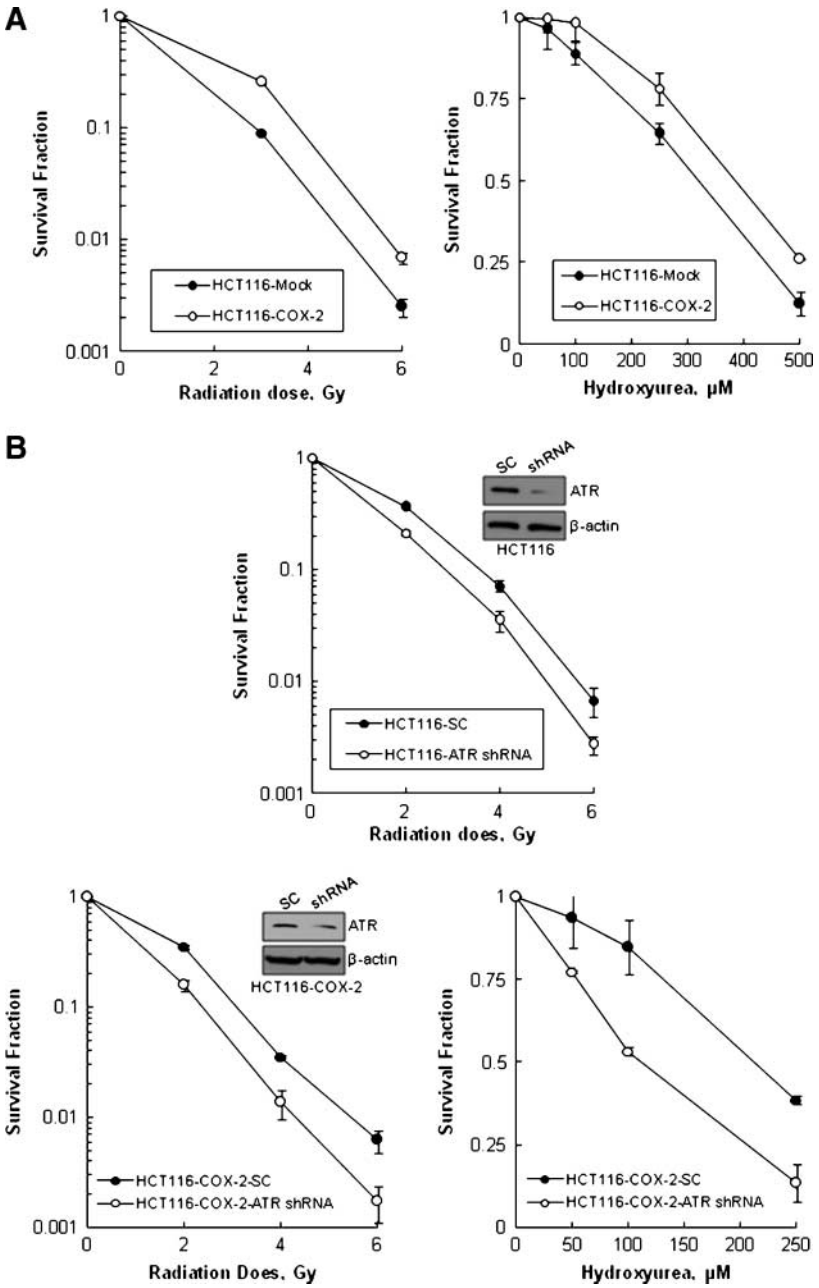

FIGURE 7. HCT116–COX-2 cells containing elevated ATR were resistant to DNA-damaging stimuli compared with HCT116-Mock cells. **A.** HCT116-Mock and HCT116–COX-2 cells were irradiated with graded doses (0, 3, and 6 Gy) of γ-rays or were incubated with hydroxyurea at indicated concentrations (0, 50, 100, 250, and 500 μmol/L). **B.** HCT116-SC and HCT116-ATR shRNA cells were irradiated with graded doses (0, 2, 4, and 6 Gy) of γ-rays (*top*). HCT116–COX-2–SC and HCT116–ATR shRNA cells were incubated with hydroxyurea at the indicated concentrations (0, 50, 100, and 250 μmol/L; *bottom*). The levels of knocked down ATR protein were analyzed by Western blotting, and survival of treated cells was determined using the clonogenic assay as described in Materials and Methods. Error bars were calculated as SE through the pooling of the results of three independent experiments.

DNA damage checkpoint pathways, and especially, activated ERK regulates ATR activity (18, 30, 39, 40).

Several reports have suggested that ERK activity regulates COX-2 expression and PD98059 inhibits cytokine or antitumor agent–induced COX-2 expression (41, 42). However, in this study, we found that COX-2 also up-regulates ERK phosphorylation and that activated ERK regulates ATR activity by increasing ATR expression. We also found that the COX-2–induced ERK activation is mediated by PGE_2. Krysan et al. also reported that PGE_2 increased ERK phosphorylation (16); this supports our results. These findings suggest that COX-2 and ERK activation may be regulated by cross-talk.

We observed that exogenously administered PGE_2 up-regulated ATR transcription and this result indicates that COX-2 induces ERK activation via PGE_2 and this activated ERK then increases ATR expression and activity at the transcriptional level. As a result, we propose that COX-2→ERK→ATR signaling pathway is constantly activated in COX-2–overexpressing cancer cells without genotoxic stresses.

We found that HCT116–COX-2 cells were resistant to IR and hydroxyurea, but HCT116-ATR shRNA cells were more sensitive to DNA-damaging agents than mock cells (Fig. 7). According to a report by Nghiem et al., ATR-WT (wild-type) overexpression may significantly increase cell survival after

Published OnlineFirst July 7, 2009; DOI: 10.1158/1541-7786.MCR-08-0493

γ-irradiation compared with control cells (Fig. 2Cin ref. 36). These data support our finding that COX-2–overexpressing cells were resistant to DNA-damaging stimuli and it may be possible through ATR up-regulation. However, we still need to investigate whether COX-2–induced ATR up-regulation directly resists DNA-damaging agents or whether this phenomenon is mediated by other indirect regulation originated from COX-2.

In conclusion, we report for the first time that COX-2 up-regulates ATR expression and activity and that this up-regulation is mediated by ERK activation by COX-2–induced PGE_2 production. Therefore, we suggest that COX-2–overexpressing cancer cells may be resistant to DNA-damaging stimuli, including IR and hydroxyurea by ATR up-regulation. Our results may aid in developing a new therapeutic target that can recover resistance to DNA-damaging agents through limiting ATR expression in COX-2–overexpressing cancer cells.

Materials and Methods

Materials

Celecoxib, a specific COX-2 inhibitor, was provided by Pfizer, Inc. PGE_2 was purchased from Cayman Chemicals. PD98059, U0126, actinomycin D, and hydroxyurea were purchased from Sigma.

Cell Culture and Reagent Treatment

HCT116 colon adenocarcinoma cells, NCI-H460 large-cell lung carcinoma cells, and A549 lung adenocarcinoma cells were purchased from the American Type Culture Collection. MOR-P lung cancer cells were kindly provided by Dr. Zhu (The University of Sheffield, Sheffield, United Kingdom). Stable COX-2–overexpressing cell lines (HCT116–COX-2 and H460–COX-2) and their mock-transfected control cells (HCT116-Mock and H460-Mock) were developed from HCT116 and NCI-H460 cells as described previously (43). COX-2 knockdown A549 cells (AS) and their mock control cells (AN) were also developed using COX-2 shRNA as previously described (29). HCT116-Mock/HCT116–COX-2 cells, H460-Mock/H460–COX-2 cells, A549, AN, AS, and MOP-R cells were cultured in RPMI 1640 (Hyclone) containing 10% fetal bovine serum (Life Technologies), 50 units/mL penicillin (Life Technologies), 50 μg/mL streptomycin (Life Technologies), 2 mmol/L L-glutamine (Life Technologies) at 37°C in an atmosphere of 5% CO_2 and 95% air. HCT116/H460-Mock and HCT116–COX-2 stable cells were incubated in a medium containing 100 μg/mL hygromycin B (Invitrogen) for 1 wk. were grown to 80% confluence in 75-cm^2 T-flasks up to 3 to 4 passages. Cells were treated with drugs (celecoxib, PGE_2, PD98059, U0126, and hydroxyurea) for the indicated times (0, 4, 8, and 24 h) and then harvested after washing twice with ice-cold PBS. Celecoxib, PGE_2, and PD98059 were solved with DMSO and final DMSO concentration in culture medium was maintained below 0.1%. A vehicle containing 0.1% DMSO was not toxic to the tested cells.

Confocal Microscopy

For immunofluorescence studies, HCT116-Mock and HCT116–COX-2 cells were grown on coverslips for 24 h, gently rinsed in 1× HBSS, and fixed with 4% paraformaldehyde in HBSS for 30 min at room temperature (44). After washing with HBSS, the cells were permeablized by incubating them with 0.1% Triton X-100 in HBSS for 30 min at room temperature. Nonspecific protein absorption was inhibited by incubation of the cells for 30 min in HBSS containing 3% bovine serum albumin, 0.2% Tween 20, and 0.2% gelatin. Cells were incubated with ATR (Calbiochem), COX-2 (BD Transduction), and γ-H2AX–specific antibodies (Upstate) diluted at 1:50 in HBSS containing 1% sucrose and 1% bovine serum albumin overnight at 4°C. After three washes with HBSS, cells were incubated for 2 h with Alexa 488 anti-mouse IgG for COX-2 (Molecular Probes) and Alexa 594 anti-rabbit IgG for ATR and γ-H2AX (Molecular Probes) diluted at 1:100, and subsequently washed with HBSS. DNA was stained with 4′,6-diamidino-2-phenylindole for 7 min at room temperature. The images were taken with confocal microscopy (Carl Zeiss).

Western Blot Analysis

Cells were lysed on ice with lysis buffer [10 mmol/L Tris-Cl (pH 8.0), 100 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L EDTA] containing 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride, 10 mmol/L NaF, and 10 mmol/L Na_3VO_4 for 15 min. The protein concentration of the cell lysate was measured by the Bradford assay (Bio-Rad Laboratories). Proteins (30 μg) were separated by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes, and probed with the following antibodies: a monoclonal antibody to COX-2, a polyclonal antibody to ATR (Santa Cruz Biotechnology), a monoclonal antibody to phospho-Chk1 (Ser^{345}), a polyclonal antibody to phospho-p53 (Ser^{15}; Cell Signaling Technology), a polyclonal antibody to ERK, and a polyclonal antibody to phospho-ERK (Cell Signaling Technology). The protein-antibody complexes were visualized with horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) for 1 h at a 1:5,000 dilution. The blots were incubated for 3 min in an ECL plus kit (GE Healthcare) and exposed to an X-ray film (AGFA). The membranes were also reprobed with a monoclonal β-actin antibody (Sigma) to normalize loading differences between the samples. All experiments were done at least in triplicate.

Reverse Transcription-PCR

Total cellular RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed for 1 h at 42°C in a reaction mixture (Clontech) that contained 1 mmol/L deoxynucleotide triphosphate, 2.5 μmol/L oligo$(dT)_{18}$, 8 mmol/L DTT, 0.4 mg/mL bovine serum albumin, 1× reverse transcriptase buffer, and 1 μg powerscript reverse transcriptase. We conducted PCR using primers (forward primer 5′-AAACTGACTCTCAGCCAACCTC-3′ and reverse primer 5′-GCATACTCATCAACTGCAAAGG-3′) for ATR in a PCR machine (GeneAmp PCR System 9700, Applied Biosystems). Cycling conditions consisted of 25 cycles, each cycle with a 30-s denaturation step at 95°C, followed by a 30-s annealing step at 55°C, and finally a 30-s extension step at 72°C. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (forward primer 5′-CAGGGCTGCTTTTAACTCTG-3′ and reverse primer 3′-GTCATGAGTCCTTCCACGATAC-5′).

Published OnlineFirst July 7, 2009; DOI: 10.1158/1541-7786.MCR-08-0493

Development of ATR Knocked Down HCT116 Stable Cell Line

The HCT116 colon cancer cells were transfected with pLKO.1 vector containing scramble sequences or ATR shRNA sequences (Open Biosystem) using FuGENE HD (Roche) for 72 h at 37°C. After transfected cells were selected with puromycin (0.2 μg/mL) for ~2 wk according to the manufacturer-provided protocol, we developed HCT116-SC and HCT116-ATR shRNA stable cell lines, and the level of knocked down ATR was detected by Western blotting.

In vitro *Clonogenic Assay*

For the radiation survival experiment, cells were serially diluted to the appropriate numbers and plated out in triplicate per data point into 100-mm dishes. The cells were incubated for 24 h at 37°C to allow attachment. Cells were irradiated with graded doses (0, 3, 6 Gy or 0, 2, 4, 6 Gy) of γ-rays using the Gammacell 3000 Elan system (MDS Nordion, Inc.). After an additional 72 h, the medium was changed and the cells were then maintained at 37°C for 6 d to allow for the formation of colonies. They were then stained with 0.5% crystal violet (Sigma) in absolute methanol to assess cell viability. The colonies were counted visually with a cutoff value of 50 viable cells. The surviving fraction was then calculated as described previously (29).

Statistical Analysis

Quantification of RT-PCR was done using Multi Gauge V3.0 (Fuji Photo Film Co.) program. The data were analyzed by Student's t test to compare the two groups and then expressed as mean ± SD. Data from the clonogenic assay were also calculated as ±SE via the pooling of the results of three independent experiments. A P value of <0.05 was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Mannino JL, Kim W, Wernick M, et al. Evidence for alternate splicing within the mRNA transcript encoding the DNA damage response kinase ATR. Gene 2001;272:35–43.

2. Bartek J, Mailand N. TOPping up ATR activity. Cell 2006;124:888–90.

3. Kumagai A, Lee J, Yoo HY, Dunphy WG. TopBP1 activates the ATR-ATRIP complex. Cell 2006;124:943–55.

4. Itakura E, Takai KK, Umeda K, et al. Amino-terminal domain of ATRIP contributes to intranuclear relocation of the ATR-ATRIP complex following DNA damage. FEBS Lett 2004;577:289–93.

5. Jazayeri A, Falck J, Lukas C, et al. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol 2006;8:37–45.

6. Wang X, Khadpe J, Hu B, Iliakis G, Wang Y. An overactivated ATR/CHK1 pathway is responsible for the prolonged G_2 accumulation in irradiated AT cells. J Biol Chem 2003;278:30869–74.

7. Sarkaria JN, Busby EC, Tibbetts RS, et al. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 1999;59:4375–82.

8. Tibbetts RS, Cortez D, Brumbaugh KM, et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev 2000;14:2989–3002.

9. Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell 2007;11:175–89.

10. Adams KE, Medhurst AL, Dart DA, Lakin ND. Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex. Oncogene 2006;25:3894–904.

11. Alderton GK, Galbiati L, Griffith E, et al. Regulation of mitotic entry by microcephalin and its overlap with ATR signalling. Nat Cell Biol 2006;8:725–33.

12. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. Science (New York) 2001;294:1713–6.

13. Lin JJ, Dutta A. ATR pathway is the primary pathway for activating G2/M checkpoint induction after re-replication. J Biol Chem 2007;282:30357–62.

14. Trifan OC, Hla T. Cyclooxygenase-2 modulates cellular growth and promotes tumorigenesis. J Cell Mol Med 2003;7:207–22.

15. Walch L, Clavarino E, Morris PL. Prostaglandin (PG) FP and EP1 receptors mediate PGF2α and PGE2 regulation of interleukin-1β expression in Leydig cell progenitors. Endocrinology 2003;144:1284–91.

16. Krysan K, Reckamp KL, Dalwadi H, et al. Prostaglandin E2 activates mitogen-activated protein kinase/Erk pathway signaling and cell proliferation in non-small cell lung cancer cells in an epidermal growth factor receptor-independent manner. Cancer Res 2005;65:6275–81.

17. McKay MM, Morrison DK. Integrating signals from RTKs to ERK/MAPK. Oncogene 2007;26:3113–21.

18. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 2007;26:3291–310.

19. Repasky GA, Zhou Y, Morita S, Der CJ. Ras-mediated intestinal epithelial cell transformation requires cyclooxygenase-2-induced prostaglandin E(2) signaling. Mol Carcinog 2007;46:958–70.

20. Sminia P, Kuipers G, Geldof A, Lafleur V, Slotman B. COX-2 inhibitors act as radiosensitizer in tumor treatment. Biomed Pharmacother 2005;59 Suppl 2:S272–5.

21. Ogunwobi OO, Beales IL. Cyclo-oxygenase-independent inhibition of apoptosis and stimulation of proliferation by leptin in human colon cancer cells. Dig Dis Sci 2007;52:1934–45.

22. Steffel J, Akhmedov A, Fahndrich C, Ruschitzka F, Luscher TF, Tanner FC. Differential effect of celecoxib on tissue factor expression in human endothelial and vascular smooth muscle cells. Biochem Biophys Res Commun 2006;349:597–603.

23. Yan Y, Black CP, Cowan KH. Irradiation-induced G2/M checkpoint response requires ERK1/2 activation. Oncogene 2007;26:4689–98.

24. Yang HM, Kim HS, Park KW, et al. Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. Circulation 2004;110:301–8.

25. Shishodia S, Koul D, Aggarwal BB. Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates TNF-induced NF-κB activation through inhibition of activation of IκBα kinase and Akt in human non-small cell lung carcinoma: correlation with suppression of COX-2 synthesis. J Immunol 2004;173:2011–22.

26. Baek SJ, Wilson LC, Lee CH, Eling TE. Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and induction of NSAID-activated gene. J Pharmacol Exp Ther 2002;301:1126–31.

27. McCubrey JA, Steelman LS, Chappell WH, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 2007;1773:1263–84.

28. Wu D, Chen B, Parihar K, et al. ERK activity facilitates activation of the S-phase DNA damage checkpoint by modulating ATR function. Oncogene 2006;25:1153–64.

29. Shin YK, Park JS, Kim HS, et al. Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. Cancer Res 2005;65:9501–9.

30. Ozeri-Galai E, Schwartz M, Rahat A, Kerem B. Interplay between ATM and ATR in the regulation of common fragile site stability. Oncogene 2007.

31. Bakkenist CJ, Kastan MB. Initiating cellular stress responses. Cell 2004;118:9–17.

32. Kumar S, Dodson GE, Trinh A, Puchalski JR, Tibbetts RS. ATR activation necessary but not sufficient for p53 induction and apoptosis in hydroxyurea-hypersensitive myeloid leukemia cells. Cell Cycle 2005;4:1667–74.

33. Leung-Pineda V, Ryan CE, Piwnica-Worms H. Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit. Mol Cell Biol 2006;26:7529–38.

34. Bennett LN, Clarke PR. Regulation of Claspin degradation by the ubiquitin-proteosome pathway during the cell cycle and in response to ATR-dependent checkpoint activation. FEBS Lett 2006;580:4176–81.

35. Niida H, Nakanishi M. DNA damage checkpoints in mammals. Mutagenesis 2006;21:3–9.

Published OnlineFirst July 7, 2009; DOI: 10.1158/1541-7786.MCR-08-0493

36. Nghiem P, Park PK, Kim YS, Desai BN, Schreiber SL. ATR is not required for p53 activation but synergizes with p53 in the replication checkpoint. J Biol Chem 2002;277:4428–34.

37. Xu B, Kim ST, Lim DS, Kastan MB. Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. Mol Cell Biol 2002;22:1049–59.

38. Soutoglou E, Misteli T. Activation of the cellular DNA damage response in the absence of DNA lesions. Science (New York) 2008;320:1507–10.

39. Yang J, Yu Y, Hamrick HE, Duerksen-Hughes PJ. ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. Carcinogenesis 2003;24:1571–80.

40. Yang J, Xu ZP, Huang Y, Hamrick HE, Duerksen-Hughes PJ, Yu YN. ATM and ATR: sensing DNA damage. World J Gastroenterol 2004;10:155–60.

41. Chae HJ, Chae SW, Reed JC, Kim HR. Salicylate regulates COX-2 expression through ERK and subsequent NF-κB activation in osteoblasts. Immunopharmacol Immunotoxicol 2004;26:75–91.

42. Funakoshi M, Tago K, Sonoda Y, Tominaga S, Kasahara T. A MEK inhibitor, PD98059 enhances IL-1-induced NF-κB activation by the enhanced and sustained degradation of IκBα. Biochem Biophys Res Commun 2001;283:248–54.

43. Kang HK, Lee E, Pyo H, Lim SJ. Cyclooxygenase-independent down-regulation of multidrug resistance-associated protein-1 expression by celecoxib in human lung cancer cells. Mol Cancer Ther 2005;4:1358–63.

44. Lee SY, Song EJ, Kim HJ, Kang HJ, Kim JH, Lee KJ. Rac1 regulates heat shock responses by reorganization of vimentin filaments: identification using MALDI-TOF MS. Cell Death Differ 2001;8:1093–102.

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Mol Cancer Res 2009;7:1158-1168. Published OnlineFirst July 7, 2009.