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Nitric Oxide Inactivates the Retinoblastoma Pathway in Chronic Inflammation

Lei Ying¹, Anne B. Hofseth¹, Darren D. Browning³, Mitzi Nagarkatti², Prakash S. Nagarkatti², and Lorne J. Hofseth¹

¹Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of South Carolina, Columbia, South Carolina

²Department of Pathology, School of Medicine, University of South Carolina, Columbia, South Carolina

³Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, Georgia

Abstract

Patients with chronic inflammatory bowel disease have a high risk of colon cancer. The molecules that initiate and promote colon cancer and the cancer pathways altered remain undefined. Here, using *in vitro* models and a mouse model of colitis, we show that nitric oxide (NO) species induce retinoblastoma protein (pRb) hyperphosphorylation and inactivation, resulting in increased proliferation through the pRb-E2F1 pathway. NO-driven pRb hyperphosphorylation occurs through soluble guanylyl cyclase/guanosine 3',5'-cyclic monophosphate signaling and is dependent on the mitogen-activated protein kinase/extracellular signal-regulated kinase MEK/ERK and phosphatidylinositol 3-kinase/AKT pathways. Our results reveal a link between NO and pRb inactivation and provide insight into molecules that can be targeted in the prevention of the inflammation-to-cancer sequence.

Introduction

A basic understanding of the mechanisms involved in the inflammation-to-cancer sequence is beginning to emerge. Key players include but are not limited to nuclear factor- κ B (NF- κ B), p53, cytokines, nitric oxide (NO) synthases, cyclooxygenases, and pattern recognition receptors (1–3). Although the onset of carcinogenesis associated with inflammation is at least in part mediated by free radical species through protein modification and DNA damage, the identification of specific free radicals and their targets remains vague. NO species are candidates due to their elevation in chronic inflammation and cancer and their ability to damage DNA and posttranslationally modify key cancer proteins (2,4). TP53, for example, is a tumor suppressor protein that is hyperphosphorylated and activated by NO in colitis (3). It is also mutated in this disease (5). Depending on levels, NO can also activate and inhibit NF- κ B activity (6,7).

Retinoblastoma protein (pRb) is also a well-known tumor suppressor. We have recently shown that the pRb protein is hyperphosphorylated and inactivated in mice and humans with colitis.

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Requests for reprints: Lorne J. Hofseth, Department of Basic Pharmaceutical Sciences, College of Pharmacy, University of South Carolina, Coker Life Sciences, Room 513C, 770 Sumter Street, Columbia, SC 29208. Phone: 803-777-6627; Fax: 803-777-8356; hofseth@cop.sc.edu.

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Because pRb hyperphosphorylation correlated with inducible NO synthase (iNOS) levels in our study (8), we hypothesized that NO drives pRb hyperphosphorylation as a mechanism toward pRb inactivation in colitis. Our elucidation of the underlying signaling pathways provides a more complete understanding of molecular basis of chronic inflammation-related carcinogenesis. Strategies targeting these newly recognized pathway interactions may prove beneficial to the chemoprevention or treatment of chronic inflammation and associated carcinogenesis.

Materials and Methods

Chemicals and reagents

Spermine NONOate (SPER/NO), U0126, and SB203580 were purchased from Alexis Biochemicals. 1*H*-[1,2,4]oxadiazole[4,3-*a*]quinoxalin-1-one (ODQ) and LY294002 were purchased from Cayman Chemical. 8-(4-Chlorophenylthio)guanosine 3',5'-cyclic monophosphate sodium salt, Rp isomer (Rp-8-pCPT-cGMP) and 8-(4-chloro-phenylthio)guanosine 3',5'-cyclic monophosphate sodium salt (8-pCPT-cGMP) were purchased from Biolog Life Science Institute. Dextran sodium sulfate (DSS) is from MP Biomedicals, Inc.

Cell culture and treatment

Four colon cancer cell lines were maintained explicitly for exploring our hypotheses: HCT116, HT29, DLD-1, and HCT15 cells. These cells, as well as the murine macrophage cell line ANA-1, which were used for coculture experiments, were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS; Biofluids), 4 mmol/L glutamine (Biofluids), 10 units/mL penicillin, and 10 µg/mL streptomycin (Biofluids). Synchronized cells (by addition of medium containing 0.1% FCS 24 h before treatment) were exposed to NO through excess L-arginine (200 µmol/L), through the NO donor SPER/NO (10 or 50 µmol/L, as indicated in text/figures), and through coculture with ANA-1 mouse macrophages (3:1 ANA-1 cells to target cells). All produce levels of nitrate and nitrite (the stable end product of NO) consistent with that which we measured in chronic mouse colitis (Table 1). For pathway inhibitor experiments, 50 µmol/L LY294002, 2 µmol/L U0126, or 10 µmol/L SB203580 were added an hour before 50 µmol/L SPER/NO treatment.

Coculture conditions

HT29 colon cancer cells were seeded at 2.5×10^6 per 150-mm culture dish 24 h before exposure to macrophages. Before the addition to HT29 cells, log-phase ANA-1 murine macrophages were activated with IFN- γ (100 units/mL for 12 h; R&D Systems). ANA-1 cells were then added to the actively growing colon cancer cells at a 3:1 ratio (ANA-1 to colon cancer cell). The coculture was incubated for 6 h before harvest. After harvest, to assure examination in target colon cancer cells only, cells were separated from ANA-1 cells with CD45⁺ magnetic beads through mass spectrometry column with MACS mini separator according to the manufacturer's instructions (Miltenyi Biotec).

Mouse model of colitis

Seven-week-old C57BL/6 iNOS^{+/+} and iNOS^{-/-} mice received either water *ad libitum* or 4% DSS for three cycles. Each cycle in the DSS group consisted of 4% DSS in drinking water for 7 days followed by a 7-day interval with normal drinking water. The nontreated group was given normal drinking water for the duration of the experiment. Following completion of the third cycle, mice were euthanized and colonic tissue was obtained. Colon tissue samples were washed with PBS and formalin fixed and paraffin embedded for pathology and immunohistochemical analysis. Immunohistochemical staining and quantification was carried out as described previously (8).

Western blot analysis and antibodies

Western blots were carried out as described previously (8,9). Antibodies used include anti-actin (Cell Signaling), anti-cyclin A (Oncogene), anti-cyclin D1 (Calbiochem), anti-pRb (Ser⁷⁸⁰), anti-pRb (Ser⁸⁰⁷ and Ser⁸¹¹), anti-pRb (4H1), anti-extracellular signal-regulated kinase (ERK) 1/2, anti-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), anti-AKT, anti-AKT (Ser⁴⁷³; Cell Signaling), anti-pRb (Thr⁸²¹ and Thr⁸²⁶; Santa Cruz Biotechnology), and anti-proliferating cell nuclear antigen (PCNA) antibody (Abcam). All antibodies were diluted at 1:500. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham. Both secondary antibodies were diluted at 1:2,000. Western blot signal was detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce) and developed onto Hyperfilm (Amersham).

Nitrate and nitrite assay

Nitrite and nitrate are the stable end products of NO metabolism and are measured in culture medium with a fluorometric assay kit as described by the vendor (Cayman Chemical).

Cell doubling time

Cells were plated on a 24-well plate (1×10^4 per well) in DMEM supplemented with 10% FBS. SPER/NO (10 μ mol/L) was added to cells 48 h after plating. Cell numbers were counted daily from day 2 to day 6. Lower doubling times are interpreted as cells growing faster.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay

Cells were plated on 96-well plate in DMEM supplemented with 10% FBS. SPER/NO (10 μ mol/L) was added to cells 48 h after plating. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to the cells at indicated time point. After 4-h incubation at 37°C, 100 μ L DMSO was added to the wells to dissolve the purple crystal. Data were read at 495 nm.

Small interfering RNA transfection

Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the instruction from the manufacturer. The cells were incubated with 150 nmol/L small interfering RNAs (siRNA) for 4 h. For E2F1 knockdown, siGENOME SMARTpool E2F1 (Dharmacon) was used. For pRb knockdown, pRb siRNA (Ambion) was used. Stealth RNA interference-negative control duplexes (Invitrogen) were applied as control siRNA in both cases. Cells were harvested or plated to 10-cm dishes 72 h after transfection. pRb siRNA sequences are as follows: sense, 5'-GGGUUGUGUCGAAAUUGGATT-3'; antisense, 5'-UCCAAUUUCGACACAACCCTG-3'.

In vitro kinase assay

FLAG epitope-tagged PKG1a was expressed in HEK293T cells and immunopurified using anti-FLAG-M2 agarose. The pure enzyme was incubated alone, with histone H1 (5 μ g positive control), with cRb fusion protein (2 μ g), in kinase buffer containing 10 μ mol/L cGMP and 10 μ Ci [³²P]ATP. Rb incubated in reaction buffer without protein kinase G (PKG) is shown as a negative control. Reactions were 30 min at 37°C. Reactions were stopped by boiling in PAGE buffer and then the whole reaction was separated on 10% gels and then subjected to autoradiography.

Statistical analysis

Mean differences in marker levels were compared by a Student's *t* test. The *P* value chosen for significance in this study was 0.05.

Results

pRb hyperphosphorylation is blunted in colons of iNOS^{-/-} mice undergoing colitis

We have previously shown that pRb is hyperphosphorylated and inactivated in mice with colitis. pRb hyperphosphorylation also correlated with iNOS induction (8). We therefore compared pRb hyperphosphorylation in iNOS^{+/+} and iNOS^{-/-} mice undergoing DSS-induced colitis. Figure 1 shows that iNOS^{-/-} mice exposed to 4% DSS for three cycles have blunted pRb hyperphosphorylation at Ser⁷⁸⁰ and Ser^{807/811} compared with iNOS^{+/+} mice. Figure 1B shows representative photomicrographs of pRb hyperphosphorylation observed in water-treated and DSS-treated mice. These results indicate that NO species are involved in pRb hyperphosphorylation. Further experiments were therefore carried out to understand the mechanism(s).

NO species induce pRb hyperphosphorylation and induction of E2F1 target molecules in multiple colon cancer cell lines

In vitro experiments were designed to examine a role for NO in pRb hyperphosphorylation and inactivation. We identified levels of nitrate and nitrite within a range which we measured in colitis (Table 1). We first used the iNOS substrate L-arginine in excess (200 μmol/L) to generate NO species. Figure 2A shows that L-arginine generates an increase in nitrate and nitrite with a concomitant induction of pRb phosphorylation at sites spanning exon 23 (Ser⁷⁸⁰ and Ser^{807/811}; Thr⁸²¹ and Thr⁸²⁶) in a time-dependent and reversible manner. This is also shown with exposure of colon cancer cells to the NO donor SPER/NO at levels that produce nitrate and nitrite concentrations similar to that which we measured in experimental colitis (50 μmol/L; Fig. 2B; Table 1). Finally, to model conditions of active inflammation, a coculture system was used. ANA-1 mouse macrophages were seeded on top of colon cancer cells at a ratio of 3 (ANA-1 cells) to 1 (colon cancer cell). These conditions also mimic levels of nitrate and nitrite measured in mouse colitis (Table 1). Figure 2C shows that, under these conditions, pRb is hyperphosphorylated at the same exon 23 sites. This response is NO dependent because the iNOS inhibitor aminoguanidine inhibits the phosphorylation of pRb by activated ANA-1 cells.

We have previously shown that, in a mouse model of colitis, pRb phosphorylation at exon 23 sites is associated with the release of E2F1 and activation of proteins downstream of pRb (8). Therefore, we also wanted to examine downstream effects by NO here. Cyclin A, cyclin D1, and PCNA are all E2F1 targets (10–12). Under all conditions, cyclin D1 and PCNA levels are increased (Fig. 2A–C). Cyclin A levels are increased after exposure to 50 μmol/L SPER/NO and cocultured activated ANA-1 cells (Fig. 2B and C) but not after L-arginine (Fig. 2A). One possibility for this latter result is that, under such conditions, NO species levels generated are too low (reflected by low nitrate/nitrite levels) for cyclin A to be induced.

NO-induced proliferation is mediated through the pRb-E2F1 pathway

To examine whether pRb is a molecular node in NO-mediated proliferation, we used siRNA for pRb. Figure 3A shows that pRb is successfully knocked down in three colon cancer cell lines. NO concentrations that produce nitrate and nitrite concentrations comparable with that which we measured in colitis (Table 1) can significantly reduce cell doubling time and increase MTT-based color (both, reflecting increased proliferation) in control (scrambled) siRNA-transfected cells but not in pRb siRNA-transfected cells (Fig. 3B and C). This result indicates that NO-induced proliferation is pRb dependent. To investigate whether this effect is pRb-E2F1 pathway dependent, we repeated the experiments with siRNA targeted to E2F1. Figure 3D and E shows a similar effect as that with siRNA to pRb. Loss of E2F1 results in a loss of a proliferative response to SPER/NO. The observation that E2F1 knockdown alone (Fig. 3D and E, *white columns*) results in increased proliferation was at first surprising. However, recent studies similarly indicate that E2F1 knockdown does not promote cellular proliferation.

Furthermore, there is some indication that other E2F family members can compensate for sibling knockout (13, 14). Taken together, these results indicate that NO-induced proliferation is both pRb and E2F1 dependent, consistent with the hypothesis that the pRb-E2F1 pathway is a molecular node in NO-driven colon cancer cell proliferation.

The soluble guanylyl cyclase/cGMP pathway mediates NO-induced pRb hyperphosphorylation

We next explored the pathway mediating pRb hyperphosphorylation and proliferation by NO. NO signaling is partly mediated through the soluble guanylyl cyclase (sGC)/cGMP pathway. Additionally, this pathway activates cGMP-dependent protein kinase (PKG; ref. 15). We therefore explored the hypothesis that this pathway is involved in pRb hyperphosphorylation. We used the pRb phosphorylated Ser⁷⁸⁰ antibody as a marker for pRb hyperphosphorylation in the present studies. Figure 4A shows that the sGC inhibitor ODQ inhibits phosphorylation caused by the NO donor SPER/NO (50 μ mol/L) in three different colon cancer cell lines. The addition of the PKG selective inhibitor Rp-8-pCPT-cGMP also inhibited NO-mediated pRb hyperphosphorylation in the colon cancer cells (Fig. 4B). To confirm that the sGC/cGMP/PKG pathway contributes to NO-induced pRb phosphorylation, we introduced 8-pCPT-cGMP, a mimetic of cGMP and selective activator of PKG, to all three tested cell lines. There was a maximal increase in pRb hyperphosphorylation between 50 and 100 μ mol/L in all cell lines, consistent with the hypothesis that the sGC/cGMP/PKG pathway is involved in pRb phosphorylation. Finally, to explore if activated PKG can phosphorylate pRb directly, an *in vitro* kinase activity assay was conducted. Results, however, were negative (Supplementary Fig. S1), indicating an intermediate step between the sGC/cGMP/PKG pathway and pRb hyperphosphorylation.

Low-concentration NO-induced pRb phosphorylation is mediated by the MEK/ERK and phosphatidylinositol 3-kinase/AKT pathways independently

Recent studies have shown that NO or cGMP can induce phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathway activation in a PKG-dependent manner (15,16). pRb phosphorylation is also associated with PI3K/AKT and MAPK/ERK kinase (MEK)/ERK1/2-mediated cyclin-dependent kinase 2 activation (17). We therefore hypothesized that NO signaling through a sGC/cGMP/PKG mechanism and subsequent pRb hyperphosphorylation is at least partially mediated by PI3K/AKT, MAPK/p38, and/or MEK/ERK1/2 pathways.

We first labeled 8-pCPT-cGMP-treated and 8-RP-pCPT-cGMP-treated cell lysates with AKT (Ser⁴⁷³) and ERK1/2 (Tyr²⁰²/Thr²⁰⁴) antibodies, respectively. Figure 5A shows the cGMP analogue treatment (8-pCPT-cGMP; labeled as cGMP in the figure) markedly increases AKT and ERK1/2 phosphorylation. Cyclin D1 levels are also increased. Alternatively, the cGMP antagonist (8-RP-pCPT-cGMP; labeled as Rp-cGMP in the figure) reverses the effect of SPER/NO on AKT and ERK1/2 phosphorylation (Fig. 5B).

We further examined these putative signaling pathways with the use of pharmacologic inhibitors. Data show that preincubation of cells with LY294002 (a PI3K inhibitor) or U0126 (a MEK inhibitor) for an hour significantly and independently attenuates NO-induced pRb phosphorylation (Fig. 5C). Inhibitors of other pathways previously shown to be downstream of PKG, such as p38 (16), were unsuccessful at inhibiting NO-induced pRb hyperphosphorylation (Supplementary Fig. S2). Taken together, results identify the PI3K/AKT and MEK/ERK1/2 pathways as independent mediators of NO-driven pRb hyperphosphorylation and inactivation.

Discussion

Rb gene mutations are common in many mesenchymal and epithelial malignancies. This includes allele loss and single nucleotide polymorphisms in sporadic colorectal cancer (18, 19). Recent studies suggest that *Rb* is down-regulated by posttranscriptional microRNA mechanisms in colon cancer (20). There is also a small amount of evidence that the pRb protein is up-regulated, hyperphosphorylated, and inactivated in this disease (21). Although the mutation load of the *Rb* gene is unknown in colitis and colon cancer associated with colitis, we have shown the protein to be up-regulated, hyperphosphorylated, and inactivated in this disease (8). Here, we show that NO species are at least partially responsible for pRb hyperphosphorylation in colitis *in vivo* and in colon epithelial cells *in vitro* and that the pRb-E2F1 pathway is a molecular node in NO-driven colon cancer cell proliferation. The mechanism of NO-driven pRb hyperphosphorylation seems to be through the sGC/cGMP/PKG signaling pathway. Our studies, in combination with several other studies implicating macrophage migration inhibitory factor and several interleukins in mediating pRb hyperphosphorylation (22–27), identify the pRb pathway as a central player in inflammatory-mediated carcinogenesis.

Studies have shown that NO species activate kinases that lead to phosphorylation of cancer-related proteins (9,28), including pRb in breast cancer cells (29). Because the biological effects of NO depend in part on its concentration and its surrounding microenvironment (3), we have carefully measured nitrate and nitrite levels (the stable end products of NO generation) in all experimental systems here. These levels were compared with levels that occur in a mouse model of colitis. pRb is hyperphosphorylated in experimental systems where nitrate and nitrite levels are similar to that which occur in colitis (Fig. 1 and Fig 2; Table 1). Under conditions where nitrate and nitrite reach levels that are substantially above that measured in colitis induced by 4% DSS, we see pRb levels depleted (Supplementary Fig. S3). Studies are ongoing to see whether this (pRb degradation) may occur in conditions where colitis is more severe than those presented here. If so, NO may play a role in pRb inactivation through hyperphosphorylation (with low but pathologic levels) or depletion (with higher levels).

NO binds and activates sGC to produce cGMP. This pathway further activates PKG. This NO/sGC/cGMP/PKG pathway has been implicated as both a protumor pathway (15,28,30–33) and an antitumor pathway (34). In colon tumor cells, accumulating evidence suggests that PKG suppresses tumorigenesis (35) and induces apoptosis (36). However, the role of this pathway in the colitis-to-cancer sequence is unknown. PKG can activate the NF- κ B pathway (37), which is a molecular node in the colitis-to-cancer sequence (1). These observations in association with evidence that pRb is inactivated through a PKG mechanism suggests that PKG might be protumorigenic in an inflamed microenvironment. Interestingly, this double-edged sword of PKG may be associated with NO concentration. Deguchi et al. (36) showed that over-expression of PKG decreases cyclin D1 levels. Alternatively, we found that low concentrations of the PKG activator (8-pCPT-cGMP) causes pRb hyperphosphorylation (Fig. 4C) and up-regulation of cyclin D1 levels (Fig. 5A), which is key to driving the cell from G₁ to S phase. We explored the hypothesis that pRb is a yet unidentified substrate for PKG. Results here are not consistent with this hypothesis (Supplementary Fig. S1). PI3K/AKT, MEK/ERK, and MAPK/p38 are three major signaling pathways that play critical roles in cellular responses to signaling molecules, such as NO (17,38). We examined these pathways due to some evidence of their interactions with PKG and/or pRb (15,39). Our results show that AKT and ERK1/2 phosphorylation increase with a cGMP analogue (Fig. 5A) and decreases with a cGMP antagonist (Fig. 5B). We also show for the first time that cyclin D1 levels elevate after cellular exposure to a cGMP analogue (Fig. 5A). Inhibitors of these two pathways show that both PI3K/AKT and MEK/ERK1/2 pathways independently relay NO/sGC/cGMP/PKG signaling to pRb hyperphosphorylation. However, the MAPK/p38 inhibitor SB203580 had no effect on NO-

induced pRb phosphorylation. Although MAPK/p38 has been recognized as an effector of activated PKG (16), this pathway does not seem to mediate NO-dependent pRb phosphorylation. Interestingly, recent studies have found that NO-induced apoptosis is not p53 dependent in colon cancer cells and that the pathways of ERK1/2 and p38 kinase are important in NO-induced apoptosis (40). Future studies can delineate whether apoptosis in colon cancer cells is associated with pRb phosphorylation and subsequent E2F activation.

In conclusion, we have shown that concentrations of NO species that produce nitrate and nitrite at similar levels to that which we have measured in mouse colitis (Table 1) lead to pRb hyperphosphorylation and inactivation. The inactivation of pRb by NO species is a key event in NO-driven hyperproliferation of human colon cancer cells. The mechanism of NO-associated hyperphosphorylation of pRb seems to be through the activation of the sGC/cGMP/PKG pathway and downstream PI3K/AKT and MEK/ERK1/2 pathways, and there are still some missing links to be investigated in the future. Evidence is emerging that both pathways provide potential cancer therapeutic targets (41–45). Because we have now established their role in NO-mediated pRb hyperphosphorylation and activation in the colon, targeting such molecules may prevent the colitis-to-cancer sequence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. pRb hyperphosphorylation is mediated by iNOS in DSS-treated mouse colons. *A*, iNOS^{-/-} and iNOS^{+/+} mice were fed 4% DSS in drinking water for three cycles. pRb phosphorylation was examined at Ser⁷⁸⁰ and Ser^{807/811} by immunohistochemistry. These sites of phosphorylation were significantly increased in DSS-treated iNOS^{+/+} mice but not in iNOS^{-/-} mice. *B*, representative photomicrographs of phosphorylated pRb at indicated sites. Magnification, $\times 400$.

Figure 2.

NO species increase pRb phosphorylation in three cell culture systems. *A*, endogenous NO produced by excess L-arginine (*L-Arg*; 200 μmol/L) causes an increase in pRb phosphorylation at Ser⁷⁸⁰, Ser⁸⁰⁷, Ser⁸¹¹, Thr⁸²¹, and Thr⁸²⁶ in HCT116 colon cancer cells. The iNOS inhibitor aminoguanidine (AG) was added to control HCT116 cells (*lanes 1–5*) to eliminate endogenous NO production. Levels of E2F1 downstream molecules, cyclins D1 (*CycD*) and PCNA, were elevated. This observation was also seen in SPER/NO-treated HT29 colon cancer cells (*B*) and HT29 colon cancer cells cocultured with ANA-1 mouse macrophages (*C*). In the latter cases (*B* and *C*), cyclin A (*CycA*) levels were also elevated. For the coculture experiment (*C*), colon cancer cells were separated from ANA-1 cells following incubation to ensure end point observation in target colon cells only.

Figure 3.

NO-mediated cell proliferation is pRb and E2F1 dependent. **A**, Western blots of pRb siRNA-transfected human colon cancer cell lines. pRb siRNA successfully lowered pRb protein levels. **B**, cell doubling time (h) in pRb siRNA-transfected and control siRNA-transfected colon cancer cells. **C**, MTT assay in pRb siRNA-transfected and control siRNA-transfected colon cancer cells. **D**, cell doubling time (h) in E2F1 siRNA-transfected and control siRNA-transfected colon cancer cells. **E**, MTT assay in E2F1 siRNA-transfected and control siRNA-transfected colon cancer cells. In most cases, SPER/NO, at concentrations consistent with that which occurs in colitis, stimulated colon cancer cell proliferation in control siRNA-treated cells but in neither pRb siRNA-transfected nor E2F1 siRNA-transfected cells.

Figure 4.

The sGC/cGMP/PKG pathway is involved in NO-mediated pRb phosphorylation. *A*, the sGC inhibitor ODQ inhibits SPER/NO-induced pRb phosphorylation in human colon cancer cell lines. *B*, the PKG inhibitor Rp-8-pCPT-cGMP also inhibits SPER/NO-induced pRb phosphorylation in human colon cancer cell lines. *C*, the cGMP mimetic 8-pCPT-cGMP stimulates pRb phosphorylation.

Figure 5.

The PI3K/AKT and MEK/ERK1/2 pathways affect NO-induced pRb phosphorylation independently. *A*, the cGMP mimetic 8-pCPT-cGMP (*cGMP*) increases AKT and ERK1/2 phosphorylation and cyclin D1 level. *B*, the cGMP antagonist Rp-8-pCPT-cGMP (*RP-cGMP*) inhibits AKT and ERK1/2 phosphorylation after SPER/NO treatment. *C*, LY294002 and U0126 effectively inhibit AKT and ERK1/2 phosphorylation, respectively. NO-induced pRb phosphorylation decreases when AKT and ERK1/2 phosphorylation are inhibited individually.

Table 1

Nitrate + nitrite produced in different systems

NO-generating system	Nitrate + nitrite produced (μmol/L)
L-arginine exposure (24 h)	5.4
Coculture system, 3:1 ANA-1 to epithelial cells (24 h)	45
SPER/NO (50 μmol/L) (24 h)	60
Water-treated mice (iNOS ^{+/+} , after three cycles)	2.9 [*]
1% DSS-treated mice (iNOS ^{+/+} , after three cycles)	7.8 [*]
4% DSS-treated mice (iNOS ^{+/+} , after three cycles)	42.1 [*]

^{*} Indicates that this is the total concentration measured in whole-colon (average, 10.1 cm) lysates.