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Report

Nitrosative stress suppresses checkpoint activation after DNA synthesis inhibition

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Abbreviations: •NO, nitric oxide; Cdk, cyclin-dependent kinase; SNCEE, *S*-nitrosocysteine ethyl ester; ATM, ataxia telangiectasia-mutated; ATR, ATM- and Rad3-related; Chk, checkpoint kinase; RNS, •NO-derived reactive species; BrdU, bromodeoxyuridine; *L*-NMMA, N^G-monomethyl-*L*-arginine monoacetate; iNOS, inducible nitric oxide synthase; ssDNA, single-stranded DNA; dsb, double-stranded DNA break

Key words: nitrosative stress, checkpoint, DNA synthesis, replication, Cdc25A, nitric oxide, Chk1, Chk2, DNA damage

DNA synthesis is promoted by the dephosphorylation and activation of cyclin-dependent kinase 2 (Cdk2) complexes by Cdc25A. Nitrosative stress suppresses Cdk2 dephosphorylation by Cdc25A in vitro and inhibits Cdc25A protein translation in cells, but the effects on S-phase progression remain unexamined. Herein we report that nitrosative stress catalyzed by inducible nitric oxide (•NO) synthase (iNOS) or the chemical nitrosant *S*-nitrosocysteine ethyl ester (SNCEE) rapidly inhibited DNA synthesis concomitant with Cdc25A loss. Surprisingly, this inhibition of DNA synthesis was refractory to ectopic expression of Cdc25A or a Cdc25-independent Cdk2 mutant. Nitrosative stress inhibited DNA synthesis without activating checkpoint signaling, thus distinguishing it from S-phase arrest mediated by other reactive •NO-derived species. The apparent lack of checkpoint activation was due to an active suppression because accumulation of pSer345-Chk1, pThr68-Chk2 and γH2AX was inhibited by nitrosative stress in cells exposed to DNA damage or replication inhibitors. We speculate that failure to activate the S-phase checkpoint in precancerous cells undergoing nitrosative stress may elevate the risk of transmitting damaged genomes to daughter cells upon cell cycle reentry.

Introduction

Accurate duplication of cellular DNA is essential for faithful transmission of the genome to daughter cells. DNA synthesis requires origin licensing, initiation, elongation and termination. Initiation and elongation phases mandate sustained Cdk2 activity (reviewed by Sclafani and Holzen).[1] Cdk2 activation requires association with cyclins, sequential phosphorylation by Wee1, Myt1 and Cdk-activating kinase, and subsequent dephosphorylation by Cdc25A.[2]

To accommodate cellular stress and DNA damage, cells have developed checkpoint pathways that inhibit Cdk2 activity, thus stopping ongoing DNA elongation and initiation of unfired origins. This checkpoint response is triggered by the activation of the phosphoinositide-3-kinase family members ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) by aberrant DNA structures.[3] ATM and ATR phosphorylate checkpoint kinases 1 and 2 (Chk1 and Chk2) on several residues including Ser345 (Chk1) and Thr68 (Chk2), activating them. Chk1 and 2 then inactivate Cdc25A, Cdc25B and Cdc25C phosphatases via destruction or relocalization, resulting in elevated inhibitory phosphorylation of Cdk2 on Thr14 and Tyr15.[2] Checkpoint activation is initiated by multiple cellular stresses including nucleotide depletion, DNA polymerase inhibition, physical blockade of replication forks, and single- and double-stranded DNA breaks (dsb).[4] Several groups have reported S-phase arrest upon exposure to •NO-generating compounds and other •NO-derived reactive species (RNS), occasionally with coincident checkpoint activation.[5,6]

Although numerous RNS inhibit S-phase progression, the mechanisms controlling DNA synthesis in cells challenged with •NO and other RNS appear distinct.[6-8] Challenge of vascular smooth muscle cells with •NO donors induces S-phase arrest with a characteristic depletion of Cdk2 activity.[7,8] Macrophage activation depletes nucleotides by •NO-inactivation of ribonucleotide reductase,[9,10] while nitrating agents induce an okadaic acid-sensitive S-phase arrest coincident with ATM activation and loss of Cdc25A, characteristic of the standard intra-S-phase checkpoint.[6]

We previously uncovered novel mechanisms regulating Cdc25A following nitrosative stress, or the aberrant accumulation of intracellular nitroso species. Cdk2 dephosphorylation by Cdc25A is inhibited by *S*-nitrosothiols, which are cellular products of nitrosative insult.[11,12] Also, nitrosative challenge suppresses Cdc25A translation, which is in contrast to the decreased Cdc25A protein stability observed after DNA damage.[2,12] As Cdc25A is rate-limiting for S-phase,[13,14] we hypothesized that translational and/or enzymatic suppression of Cdc25A would blunt DNA synthesis in cells

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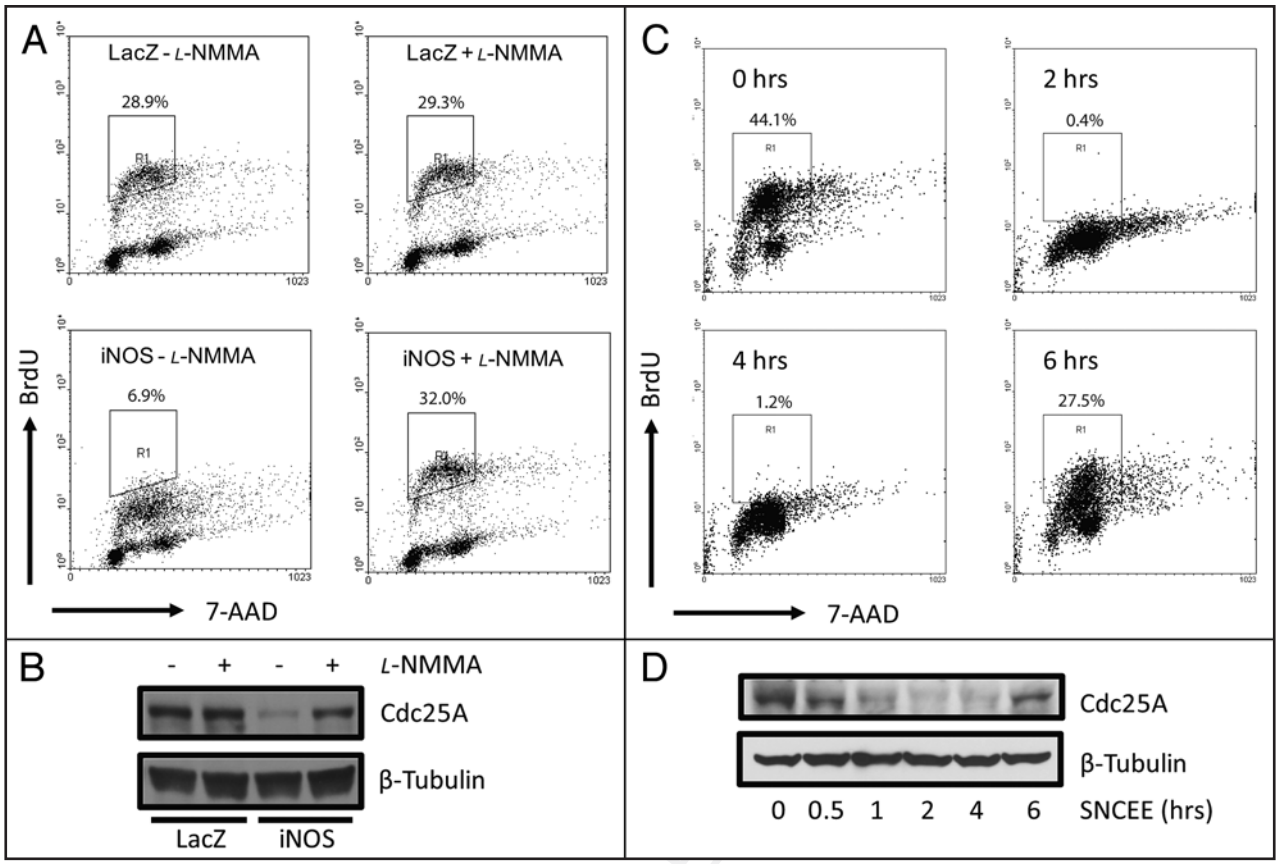

Figure 1. Nitrosative stress suppressed Cdc25A expression and DNA synthesis concomitantly. (A) HCT116 cells were infected with 10 MOI of Ad-LacZ or Ad-hiNOS for one hour before addition of medium with or without 1 mM L-NMMA. Twenty-four hours later, cells were harvested and prepared for flow cytometry. Representative (N = 3) dotplots for each experiment are shown. Percentages represent the proportion of cells that are BrdU-positive. (B) Cell lysates from duplicate plates infected as in (A) were subjected to Western blotting for the indicated proteins. (C) HCT116 cells were treated with 100 μM SNCEE for the indicated times and prepared for flow cytometry. (D) HCT116 cells were treated with 100 μM SNCEE for the indicated times and lysates were prepared and analyzed for Cdc25A and β-tubulin levels by Western blotting.

experiencing nitrosative stress because of deficient Cdk2 activity. In this report, we found that nitrosative stress inhibited DNA synthesis concomitant with Cdc25A loss, but was not limited by the levels of Cdc25A or the activity of Cdk2. Furthermore, DNA synthesis inhibition following nitrosative stress occurred without activation of the S-phase checkpoint machinery. This failure to activate checkpoint was due to an active suppression of checkpoint initiation, as induction of nitrosative stress in cells exposed to a variety of DNA replication inhibitors blocked checkpoint activation almost completely. Together, these results provide insight into the mechanism of DNA synthesis inhibition under nitrosative stress and raise the concern that DNA damage accrued in cells experiencing nitrosative challenge may go unrepaired due to deficient checkpoint activation, resulting in mutagenesis.

Results

Nitrosative stress suppressed DNA synthesis coincident with Cdc25A loss. Because Cdc25A controls S-phase progression via its activation of Cdk2 complexes[13,14] and nitrosative stress suppresses Cdc25A protein levels and activity,[12] we probed the effects of nitrosative stress on S-phase progression. We found that DNA synthesis was suppressed in two models of nitrosative stress: chemical induction with SNCEE and biological induction via iNOS-mediated •NO overproduction (Fig. 1A and C). •NO formation by iNOS was essential for S-phase suppression, as the NOS inhibitor L-NMMA restored DNA synthesis to control levels in iNOS-expressing cells (Fig. 1A). Similarly, 100 μM SNCEE suppressed BrdU incorporation in a time-dependent fashion, with maximal inhibition by two hours and rebounding by 6 hours post-treatment (Fig. 1C). DNA synthesis was not affected by decomposed SNCEE (Suppl. Fig. S1), indicating this effect was specific for the intact nitrosant. This S-phase arrest is consistent with the half-life of SNCEE in cell culture medium at 37°C (approx. two hours, data not shown). Suppression of DNA synthesis was mirrored by Cdc25A loss in both cases (Fig. 1B and D). Together, these results indicate that nitrosative stress suppressed DNA synthesis concomitant with Cdc25A loss.

DNA synthesis inhibition was refractory to Cdc25A expression in cells experiencing nitrosative stress. Because Cdc25A levels were decreased with kinetics similar to those of DNA synthesis inhibition (Fig. 1B), we probed whether suppression of DNA synthesis resulted from deficient Cdc25A and therefore Cdk2 activity (Fig. 2). We expressed supraphysiological levels of Cdc25A in cells challenged with nitrosative stress generated either by iNOS (Fig. 2A) or SNCEE (Fig. 2C), and measured DNA synthesis. Although Cdc25A was elevated (Fig. 2B and D), DNA synthesis was not restored in iNOS-expressing cells (Fig. 2A), nor was the onset, duration, intensity, or

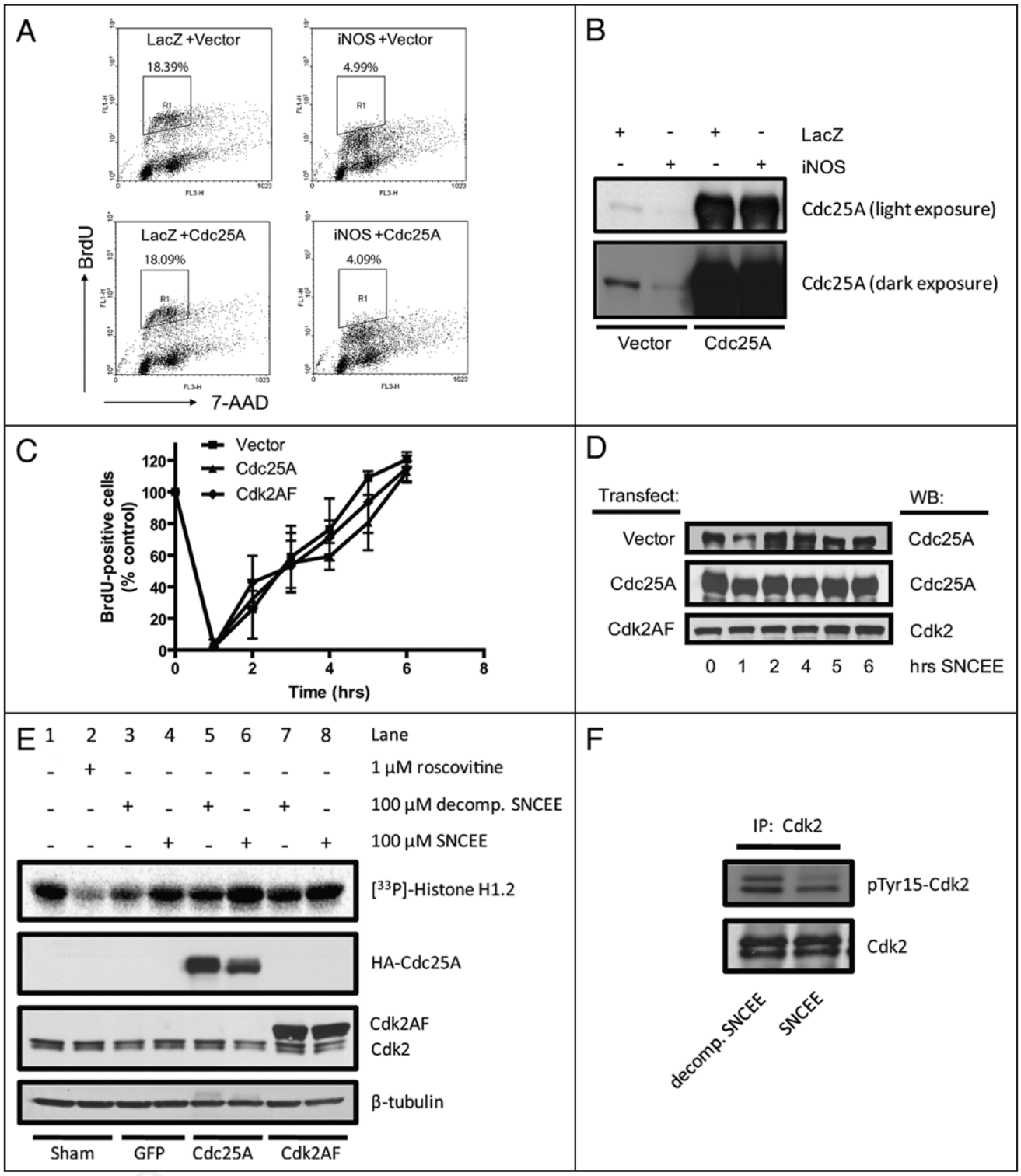

Figure 2. Nitrosative stress-induced DNA synthesis inhibition was not limited by Cdk2 activity. (A and B) HCT116 cells were transfected with vector control or pCMV-HA-Cdc25A. After 24 hours, cells were infected with the indicated adenoviruses and harvested 24 hours post-infection for flow cytometric analysis (A) or Western blotting (B). (C and D), HCT116 cells were transfected with the indicated vectors, and after 24 hours were treated with 100 μM SNCEE for the indicated times. Cells were then harvested for flow cytometry (C) or immunoblotting with the indicated antibodies (D). Vertical bars are SE, N = 3. (E) Cells were transfected with vectors encoding the indicated proteins. After 24 hours, cells were treated for one hour with the indicated compounds and cell lysates were prepared for Western blotting (HA-Cdc25A, Cdk2/Cdk2AF and β-tubulin panels) or measurement of kinase activity ([^{33}P]-histone H1.2). (F) HCT116 cells were treated for 1 hour with 100 μM decomposed or fresh SNCEE before harvesting. Cdk2 was immunoprecipitated and subjected to Western blotting with the indicated antibodies.

recovery from DNA synthesis inhibition altered in SNCEE-treated cells (Fig. 2C).

Cdk2 activity was not limiting for DNA synthesis in cells experiencing nitrosative stress. Because nitrosative stress can enzymatically inactivate Cdc25A,[12] we considered the possibility that Cdc25A expressed in nitrosatively-challenged cells might be inactive and unable to activate Cdk2. We expressed a mutated Cdk2 that does not require Cdc25A for activation (Cdk2AF)[15] and measured its effects on DNA synthesis in cells challenged with SNCEE. Although expression of Cdk2AF elevated Cdk2 levels (Fig. 2D), DNA synthesis was not restored (Fig. 2C). To assure that Cdc25A and Cdk2AF expression elevated Cdk2 activity, we measured Cdk2 activity from cells following nitrosative challenge. As shown in Figure 2E, Cdk2 rapidly phosphorylated histone H1.2, and this effect was blocked by the Cdk inhibitor roscovitine (lane 1 vs. 2). As expected, expression of Cdc25A or Cdk2AF elevated Cdk2 activity in both decomp. SNCEE- and SNCEE-treated cells (lane 3 vs. 5 and 7; lane 4 vs. 6 and 8). Although transfection and/or exposure to decomp. SNCEE partially reduced Cdk2 activity (lane 1 vs. lane 3), comparison of Cdk2 activity from SNCEE-treated cells to that from decomp. SNCEE-treated cells (lane 3 vs. 5 and 7; lane 4 vs. 6 and 8) indicates that SNCEE treatment did not compromise Cdk2 activity; rather Cdk2 activity was surprisingly increased following induction of nitrosative stress and correlated with pTyr15-Cdk2 dephosphorylation (Fig. 2F). We hypothesize this may result from dual suppression of Cdc25A[12] and Wee1 or Myt1 by nitrosative stress, as SNCEE had no direct effect on Cdk2 activity in vitro (Suppl. Fig. S2). Together, these results indicated that nitrosative stress inhibited DNA synthesis without attenuating Cdk2 activity and suggested that Cdk2 activity was not limiting for DNA synthesis in cells undergoing nitrosative stress.

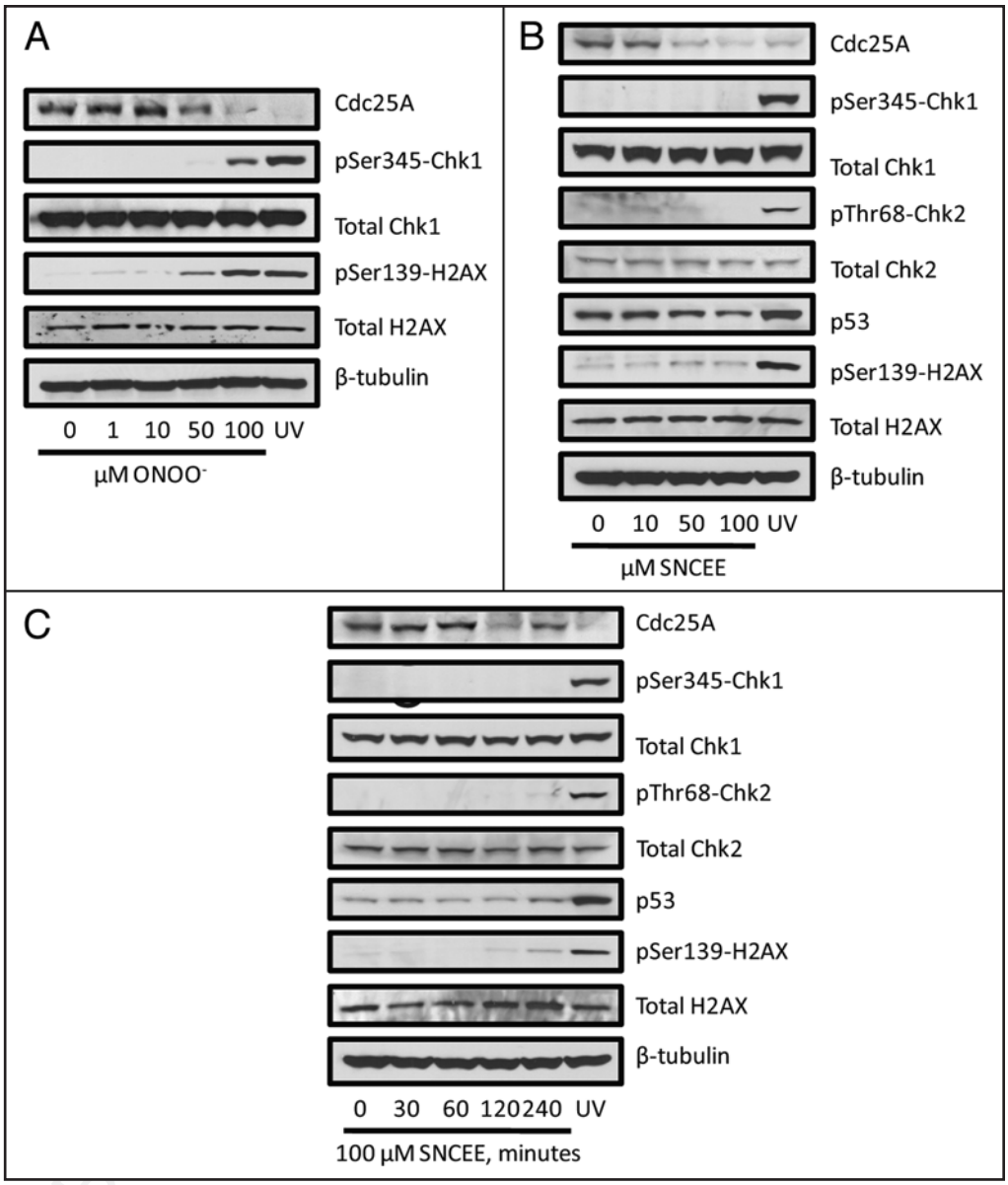

Figure 3. Nitrosative stress did not induce checkpoint activation. (A) HCT116 cells were treated with the indicated concentrations of $ONOO^-$ or exposed to 60 J/m^2 UV for two hours before harvesting and immunoblotting with the indicated antibodies. (B) HCT116 cells were treated for two hours with the indicated concentrations of SNCEE or 60 J/m^2 UV before harvesting for Western blotting. (C) HCT116 cells were treated with 100 μM SNCEE for the indicated times or with 60 J/m^2 UV for two hours and harvested as above. Lysates were probed for the indicated proteins by Western blotting.

The S-phase checkpoint was not activated following nitrosative challenge. Abrupt suppression of DNA synthesis in response to stress is mediated via the activation of the ATM and ATR kinases in response to aberrant DNA structures.[3,4] These kinases in turn phosphorylate the effector kinases Chk1 and Chk2, the proposed marker of damaged DNA, histone γH2AX,[19] and stabilize p53 tumor suppressor. Nitrating agents activate ATM,[6] which can in turn phosphorylate and activate the Chk1 and/or Chk2 kinases, which degrade Cdc25A. We examined whether γH2AX, Chk1 or Chk2 were phosphorylated and whether p53 was stabilized to query checkpoint activation following SNCEE or the nitrating agent $ONOO^-$. Figure 3A shows increased γH2AX and pSer345-Chk1, and decreased Cdc25A with increasing $ONOO^-$ treatment as was observed for other nitrating agents.[6] In contrast to $ONOO^-$ and UV irradiation, concentrations of SNCEE that almost completely inhibited DNA synthesis (Figs. 1C and 2B) yielded no significant induction of γH2AX, pSer345-Chk1, or pThr68-Chk2 phosphorylation two hours after SNCEE treatment and did not stabilize p53 (Fig. 3B).

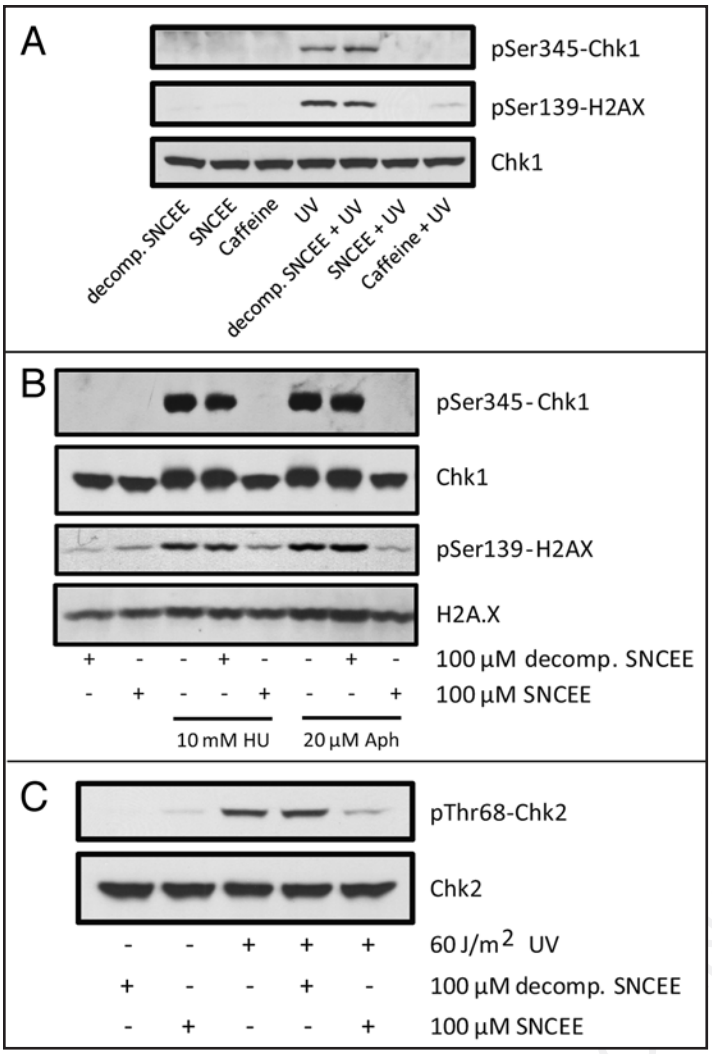

Figure 4. Nitrosative stress suppressed checkpoint activation in response to diverse DNA replication inhibitors. (A) HCT116 cells were irradiated with 60 J/m^2 UV and immediately treated with 100 μM decomp. SNCEE, 100 μM SNCEE, or 10 mM caffeine. Two hours later, cells were harvested and subjected to immunoblotting with the indicated antibodies. (B) HCT116 cells were exposed to vehicle, 10 mM hydroxyurea (HU), 20 μM aphidicolin (Aph), or 60 J/m^2 UV and either 100 μM decomposed or fresh SNCEE. Two hours later, cells were harvested for Western blotting with the indicated antibodies. (C), HCT116 cells were irradiated or not with 100 J/m^2 UV, and immediately treated either with nothing, 100 μM decomposed or fresh SNCEE as indicated. Four hours later, cells were harvested for Western blotting and probed with antibodies to pThr68-Chk2 or Chk2.

We considered the possibility that the checkpoint was activated prior to DNA synthesis inhibition. As shown in Figure 3C, however, there was no accumulation of γH2AX, pSer345-Chk1, pThr68-Chk2 or p53 at time points before or after DNA synthesis inhibition (Figs. 3C and 1C), which indicated that checkpoint activation was not required for DNA synthesis inhibition in SNCEE-treated cells. Together, these results suggest that nitrosative stress inhibited DNA synthesis without causing checkpoint initiation.

Nitrosative stress suppressed checkpoint activation following mechanistically distinct replication stresses. Nitrosative stress either failed to trigger checkpoint signaling following S-phase arrest or actively suppressed it. Thus, we examined whether nitrosative stress could quench checkpoint signaling induced by UV-irradiation. Figure 4A shows that exposure to SNCEE immediately following UV suppressed pSer345-Chk1 and γH2AX as efficiently as the ATM/ATR inhibitor caffeine. We next inquired whether checkpoint suppression by nitrosative stress was specific to UV. We treated cells simultaneously with decomposed SNCEE or SNCEE and with the DNA polymerase inhibitor aphidicolin or the ribonucleotide reductase inhibitor hydroxyurea (Fig. 4B). As observed for UV, SNCEE suppressed pSer345-Chk1 and pSer139-H2AX following either aphidicolin or hydroxurea, indicating that SNCEE suppressed checkpoint activation in response to mechanistically distinct S-phase antagonists. UV can also activate Chk2.[20] We thus queried whether nitrosative stress could also block activation of Chk2 following UV. We observed pThr68-Chk2 accumulation in UV-treated cells exposed to decomposed SNCEE but it was attenuated substantially in UV-treated cells exposed to SNCEE (Fig. 4C), indicating that nitrosative stress could suppress activation of both Chk1 and Chk2.

Discussion

Checkpoint signaling restrains cell cycle advancement when DNA damage is present; thus checkpoint deficiency represents a potent hazard to the faithful transmission of the genetic code. Nitrosative stress occurs in a variety of cancer-prone, chronic inflammatory diseases,[21] and disruption of checkpoint in these tissues could promote mutagenesis ultimately leading to tumorigenesis.[22]

The mechanism by which nitrosative stress causes DNA synthesis inhibition is unknown. Although Cdc25A protein levels were decreased, Cdk2 activity was surprisingly elevated under nitrosating conditions and correlated with Tyr15 dephosphorylation (Fig. 2E and F). This distinguishes DNA synthesis inhibition in nitrosatively-challenged cells from that of vascular smooth muscle cells exposed to •NO donors, which display decreased Cdk2 activity.[7,8] Also, our experiments indicated that Tyr15 hyperphosphorylation of Cdk2 was not necessary for suppression of DNA synthesis, which is in agreement with a recent report that Tyr15 phosphorylation of the yeast homolog cdc2 is not necessary for S-phase checkpoint.[23] In contrast to cells exposed to ionizing radiation,[24] bolstering cellular Cdk2 activity under nitrosative stress by overexpressing Cdc25A or Cdk2AF did not bypass DNA synthesis inhibition. This failure to bypass DNA synthesis inhibition by ectopic Cdc25A has also been reported in response to the DNA-damaging carcinogen benzo[a]pyrene diol epoxide,[25] hyperosmotic stress and UV.[26] This discrepancy is likely due to the type of checkpoint signaling (dsb-induced vs. other), as the dsb-induced checkpoint, which is activated strongly by IR, is known to be dependent upon Tyr15 Cdk2 phosphorylation, whereas checkpoint signaling by other stresses are independent.[24,25,27,28]

Others have reported that •NO overproduction in macrophages can quench ribonucleotide reductase activity, but we were not able to restore DNA synthesis with deoxynucleosides in cells exposed to SNCEE (Suppl. Fig. S3), indicating that nucleoside reduction was not the limiting factor. Also, our preliminary experiments indicate that DNA synthesis inhibition by nitrosative stress was not affected by ATM/ATR inhibition (Suppl. Fig. S4). Thus, S-phase arrest in cells experiencing nitrosative stress appears distinct from that induced by macrophage activation.[9,10]

It is of note that we did not observe complete suppression of DNA synthesis in cells experiencing iNOS-induced nitrosative stress

as we did in cells treated with 100 μM SNCEE (Fig. 1A vs. C), even at other timepoints following iNOS expression (data not shown). Whether this simply represented a "dosage" effect or a distinct mechanism is unclear, although our preliminary experiments indicate that Chk1 is not activated while DNA synthesis is attenuated in iNOS-expressing cells. We found previously that iNOS expression produced approximately 50 μM of the stable •NO endproducts NO_2^- and NO_3^- by 24 hours.[12] Although the value is similar in magnitude to the concentration of SNCEE used in these studies, the rate of •NO production by iNOS was likely lower than the rate of •NO release by SNCEE. We therefore cannot exclude disparate rates of exposure to •NO or RNS as responsible for this effect. Similarly, differences in subcellular localization of nitrosative stress may account for the incomplete inhibition of DNA synthesis in iNOS-expressing cells. It is possible that iNOS expression was restricted to distinct subcellular areas and thus may have produced localized nitrosative stress, whereas SNCEE is membrane-permeable[29] and therefore could affect all cellular compartments. Further experiments will be necessary to clarify this variance in DNA synthesis inhibition following nitrosative stress.

How does nitrosative stress suppress checkpoint activation? Checkpoint signaling in response to UV, hydroxurea and aphidicolin is initiated by ATR. ATR activation requires tethering to RPA-coated single stranded DNA (ssDNA) by ATRIP and recruitment of TopBP1 by the 9-1-1 complex, which is also RPA-dependent.[4] The binding of ssDNA by RPA is redox-sensitive and requires reduced Cys486.[30] Nitrosative stress induces *S*-nitrosothiol formation and thiol oxidation,[12] raising the possibility that ssDNA-binding by RPA could be suppressed by SNCEE, resulting in delocalization of ATR from stalled forks. This could also explain the suppression of Chk2 activation in cells experiencing nitrosative stress (Fig. 4C), as deficient ATR activation results in deficient ATM-dependent Chk2 phosphorylation in response to UV.[20]

In conclusion, these data distinguish DNA synthesis inhibition by nitrosative stress from that induced by other RNS and raise the possibility that DNA damage accrued during nitrosative challenge could go undetected, resulting in accumulation of mutations.

Materials and Methods

Cell culture and drug treatments. HCT116 colon adenocarcinoma cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin in a humidified 37°C incubator with 5% CO_2. All compounds were dissolved either in medium or DMSO before addition to cells unless otherwise indicated.

Reagents and chemicals. N^G-monomethyl-*L*-arginine monoacetate (*L*-NMMA), roscovitine, recombinant human histone H1.2, caffeine and hydroxyurea (HU) were from Calbiochem (La Jolla, CA). [γ-^{33}P]-ATP was from GE Healthcare Lifesciences (Piscataway, NJ). Peroxynitrite was purchased from Cayman Chemical (Ann Arbor, MI), and was diluted into 0.3 M NaOH and quantified using its extinction coefficient (ε = 1670 $M^{-1}cm^{-1}$ at 302 nm) immediately before use. Aphidicolin, ethyl nitrite and cysteine ethyl ester hydrochloride were from Sigma (St. Louis, MO). SNCEE and decomposed SNCEE (decomp. SNCEE) were synthesized exactly as reported previously.[12]

Plasmid transfection. HCT116 cells were transfected at approximately 50% density with empty vectors, or vectors encoding GFP (pEGFP-C_2, Clontech, Mountain View, CA), HA-tagged Cdc25A (pCMV-HA-Cdc25A),[12] or Cdk2AF (pcDL298α-Cdk2AF-HA)[15] using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In studies requiring subsequent adenoviral infection (see below), cells were replated 8 hours post-transfection (final cell density 30–40%) and allowed to reattach overnight before infection the following morning.

Adenoviral infection. HCT116 cells or HCT116 cells transfected 24 hours previously as described above were exposed to 1.2 mL of PBS containing 10 MOI of either Ad-LacZ or Ad-iNOS[16] for one hour at 37°C. Medium with or without *L*-NMMA (final concentration = 1 mM) was then added to each dish.

Flow cytometry. DNA synthesis was measured by bromodeoxyuridine (BrdU) incorporation using the FITC-BrdU flow cytometry kit from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions. Cells were incubated with 10 μM BrdU in complete medium for 30 minutes before harvesting and preparation according to the manufacturuer's instructions. Cell fluorescence was measured in the FITC and PI channels with appropriate compensation using a BD FACScalibur flow cytometer (BD, San Diego, CA) or a Guava EasyCyte flow cytometer (Guava Technologies, Inc., Hayward, CA). Data analysis was performed using Cytosoft 5.0.2 (Guava Technologies, Inc.,) and WinMDI 2.8.

Immunoblotting. Cells were harvested in a modified radioimmunoprecipitation buffer[17] and either sonicated at 50% amplitude for 6 x 2 seconds on ice with a 2 second pause between pulses using a GEX-130 ultrasonic processor with a VC-50 2 mm microtip (Gene Q, Montreal, Quebec, Canada) or incubated on ice for 30 minutes with frequent vortexing. Lysates were cleared by centrifugation at 13,000 xg for 15 min. Protein content was determined by the method of Bradford. Total cell lysates (30–50 μg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes using the iBlot (Invitrogen) according to the manufacturer's directions. Antibodies against Cdc25A (sc-7389), Cdk2 (sc-163), Chk1 (sc-8408) and Chk2 (sc-17747) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pTyr15-Cdk (#9111), pSer345-Chk1 (#2341), pSer139-H2AX (#2577), p53 (#9282), pThr68-Chk2 (#2661) and total H2AX (#2595) were from Cell Signaling Technologies (Danvers, MA). Antibodies to β-tubulin (#CLT9003) were from Cedarlane Laboratories (Burlington, NC), and the HA antibodies (HA.11) were from Covance (Princeton, NJ). Bound primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and proteins were visualized using Pierce enhanced chemiluminescence Western blotting substrate (Pierce Biotechnology).

Immunoprecipitation and kinase assays. For Cdk2 immunoprecipitation, 200–500 μg of cell lysates diluted to 1 mL total volume in modified RIPA buffer were precleared with 50 μL of agarose-conjugated normal rabbit IgG (sc-2345, Santa Cruz Biotechnology) for one hour at 4°C on a rotating mixer. After centrifugation at 1,500 xg, the supernatant was transferred to a tube containing 100 μL of agarose-conjugated Cdk2 antibodies (sc-163-AC, Santa Cruz Biotechnologies) and mixed overnight at 4°C. The following morning, the samples were centrifuged as above, and the pellets were washed twice with ice-cold modified RIPA buffer. For subsequent

Western blotting, beads were boiled in 50 μL of Laemmli buffer and analyzed as above.

Cdk2 kinase assays were carried out essentially as described previously[18] except the substrate was human histone H1.2. Briefly, after Cdk2 was immunoprecipitated as above, beads were washed twice in modified RIPA buffer and once with Cdk2 kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT), and then incubated in 50 μL of Cdk2 kinase buffer containing 1 μg/μL histone H1.2. Kinase reactions were initiated by the addition of 1 μM ATP containing 5 μCi [γ-^{33}P]-ATP, and were carried out for 5 minutes at 30°C. Reactions were terminated by the addition of Laemmli buffer and boiling, and 45 μL of the reaction mixture was subjected to SDS-PAGE. Gels were exposed to intensifying screens overnight and visualized using the Storm Imaging System (GE Healthcare).

UV irradiation. HCT116 cells were washed once with phosphate-buffered saline and irradiated with a UVC Crosslinker (Stratagene, La Jolla, CA), followed by addition of fresh complete medium.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/TomkoCC8-2-Sup.pdf

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