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Multimodal Control of Cdc25A by Nitrosative Stress

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Abstract

Cdc25A propels cell cycle progression, is overexpressed in numerous human cancers, and possesses oncogenic and antiapoptotic activities. Reactive oxygen species, such as hydrogen peroxide, regulate Cdc25A, but the physiologic and pathologic effects of nitric oxide (•NO) and •NO-derived reactive species are not well defined. Herein, we report novel independent mechanisms governing Cdc25A in response to nitrosative insult. We observed direct and rapid inhibition of Cdc25A phosphatase activity after *in vitro* treatment with the low molecular mass cell-permeable *S*-nitrosothiol *S*-nitrosocysteine ethyl ester (SNCEE). In addition, treatment of cancer cells with SNCEE induced nitrosative stress and decreased Cdc25A protein levels in a time-dependent and concentration-dependent manner. Similarly, iNOS-derived •NO was sufficient to suppress Cdc25A expression, consistent with its role in mediating nitrosative stress. Whereas a decrease in Cdc25A half-life was not observed in response to SNCEE, we found the translational regulator eukaryotic initiation factor 2α (eIF2α) was hyperphosphorylated and total protein translation was decreased with kinetics consistent with Cdc25A loss. Inhibition of eIF2α decreased Cdc25A levels, supporting the hypothesis that SNCEE suppressed Cdc25A translation through inhibition of eIF2α. Nitrosative stress decreased the Cdc25A-bound fraction of apoptosis signal-regulating kinase-1 (ASK-1) and sensitized cells to apoptosis induced by the ASK-1–activating chemotherapeutic *cis*-diaminedichloroplatinum (II), suggesting that nitrosative stress–induced suppression of Cdc25A primed cells for ASK-1–dependent apoptosis. Together these data reveal novel •NO-dependent enzymatic and translational mechanisms controlling Cdc25A, and implicate Cdc25A as a mediator of •NO-dependent apoptotic signaling. [Cancer Res 2008;68(18):7457–65]

Introduction

It is well accepted that the Cdc25 dual-specificity phosphatases catalyze progression through the cell cycle by dephosphorylating and activating the cyclin-dependent kinases (Cdk; ref. 1). The three mammalian isoforms, Cdc25A, Cdc25B, and Cdc25C, cumulatively contribute to progression into and through mitosis by dephosphorylating the Cdk1/cyclin B complex. Recent evidence suggests these phosphatases act on distinctly localized cellular pools of Cdk substrates and other currently unidentified substrates to control the timing and distribution of mitotic activities (2, 3). Whereas mitotic progression is a cumulative effort by these phosphatases, Cdc25A is the primary regulator of the G_1-S transition and S-phase progression via activation of Cdk2 complexes (1). Cdc25A is overexpressed in numerous human cancers (4) and suppresses apoptosis by binding to and inhibiting apoptosis signal-regulating kinase-1 (ASK-1; ref. 5). Cdc25A overexpression also drives aberrant progression through the G_1-S transition (1), induces DNA damage (6), and promotes radioresistant DNA synthesis in irradiated cells (7). Thus, understanding mechanisms controlling Cdc25A levels and activity are of basic biological and therapeutic interest.

The observation that oxidants regulate the activity of the Cdc25 phosphatases provides a potential linkage between cell redox status and cell cycle progression. *In vitro*, H_2O_2 inhibits Cdc25 activity by oxidation of the catalytic cysteine (8). The Cdc25C catalytic cysteine is oxidized to an intramolecular disulfide in H_2O_2-treated cells, although the impact of this phosphatase inactivation on cell cycle regulation remains unclear (9). H_2O_2 also decreases Cdc25A expression in HeLa cells, although the mechanism remains unidentified (10).

The effects of •NO and other reactive •NO-derived species (RNS) on Cdc25A are even less well defined, although they are physiologically and pathologically important. Cdc25A expression decreases in response to the nitrating agents $•NO_2$ and SIN-1 (11). The loss of Cdc25A is okadaic acid–sensitive and parallels ATM kinase hyperphosphorylation, which decreases Cdc25A protein half-life through activation of Chk2, subsequent Cdc25A phosphorylation, and proteasomal degradation (7). This study however did not examine the effects of •NO or *S*-nitrosating agents on Cdc25A.

•NO is a diatomic free radical, generated by nitric oxide synthases (NOS), which acts as a signaling molecule in numerous critical cellular processes, including vasodilation, synaptic transmission, and inflammation. Vasodilation and synaptic transmission are generally modulated by the production of low quantities of •NO by eNOS and nNOS, respectively. Inflammatory responses, bacterial infection, and tumorigenesis induce the production of greater concentrations of iNOS-derived •NO and secondary nitrosating, nitrating, and oxidizing species (12–14). Production of high quantities of •NO by iNOS induces nitrosative stress, which is characterized by failure to regulate the concentration of intracellular nitroso-species (15, 16). Expression of iNOS is observed in both cancerous tissues and precancerous lesions of chronic inflammatory diseases (13).

Generation of protein-associated *S*-nitroso species or *S*-nitrosation has recently gained attention as a cellular signaling mechanism (17). *S*-nitrosation is a reversible modification capable of altering protein function and cell signaling. Thus, understanding the cellular targets of protein *S*-nitrosation in cells may define effects of nitrosative stress *in vivo*. We therefore aimed to characterize the role of nitrosative stimuli on Cdc25A activity in cancer cells.

We now report biochemical mechanisms regulating Cdc25A in response to nitrosative insult. *S*-nitrosothiols rapidly inhibited the *in vitro* phosphatase activity of Cdc25A toward both artificial and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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endogenous substrates. Induction of cellular nitrosative stress using the cell-permeable nitrosating agent *S*-nitrosothiol *S*-nitrosocysteine ethyl ester (SNCEE) suppressed Cdc25A protein levels via hyperphosphorylation and inhibition of the translational regulator eukaryotic initiation factor 2α (eIF2α). Generation of •NO from iNOS also decreased Cdc25A protein levels, consistent with a role of iNOS in nitrosative stress induction. Nitrosative stress decreased the Cdc25A-bound fraction of ASK-1 and synergized with *cis*-diaminedichloroplatinum (II) (CDDP) to induce apoptotic cell death, consistent with a model where Cdc25A suppression by nitrosative stress primes cells for ASK-1–mediated cell death. Together, these results describe blunting of Cdc25A levels and activity in response to nitrosative insult and implicate Cdc25A suppression as a cellular priming event for ASK-1–dependent apoptosis.

Materials and Methods

Cell culture and drug treatments. HCT116 cells (a gift from Dr. Bert Vogelstein of the Johns Hopkins University) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, and 2 mmol/L L-glutamine in a humidified incubator at 37°C with 5% CO_2. HeLa cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin/streptomycin, and 2 mmol/L L-glutamine in a humidified incubator at 37°C with 5% CO_2. Compounds were dissolved into DMSO or directly into medium and added to cells for the indicated times.

Reagents. All compounds were from Sigma-Aldrich unless otherwise noted. Cycloheximide (CHX), DTT, glutathione, Hoechst 33342, N^G-monomethyl-L-arginine monoacetate (L-NMMA), *N*-acetyl-Leu-Leu-norleu-CHO (LLnL), and salubrinal were from Calbiochem. *S*-nitrosoglutathione and nitrotyrosine bovine serum albumin were from Cayman Chemical. The pCMV-HA-Cdc25A plasmid encoding Cdc25A was generated by cloning the human *CDC25A* cDNA into the *Eco*R1 and *Xho*I sites of the pCMV-HA vector (Clontech). The pcDNA3-Cdc25A vector encoding untagged Cdc25A (18) and the pcDNA3-HA-ASK-1 vector (5) were described previously. SNCEE was synthesized and quantified using its extinction coefficient in methanol [1,019 $(mol/L)^{-1}$ cm^{-1} at 343 nm], as previously described (19). Light homolyzes the *S*-nitrosothiol (RSNO) S-N bond, releasing •NO and thiyl radicals, which rapidly recombine in the absence of competing species to generate disulfides (20). •NO rapidly autooxidizes in the presence of O_2 and H_2O to nitrite (16). Thus, the products of SNCEE decomposition are L-cysteine ethyl ester disulfide and nitrite. SNCEE was decomposed by incubation in clear conical vials at ambient temperature under laboratory lighting for ≥24 h to generate decomposed SNCEE. We verified

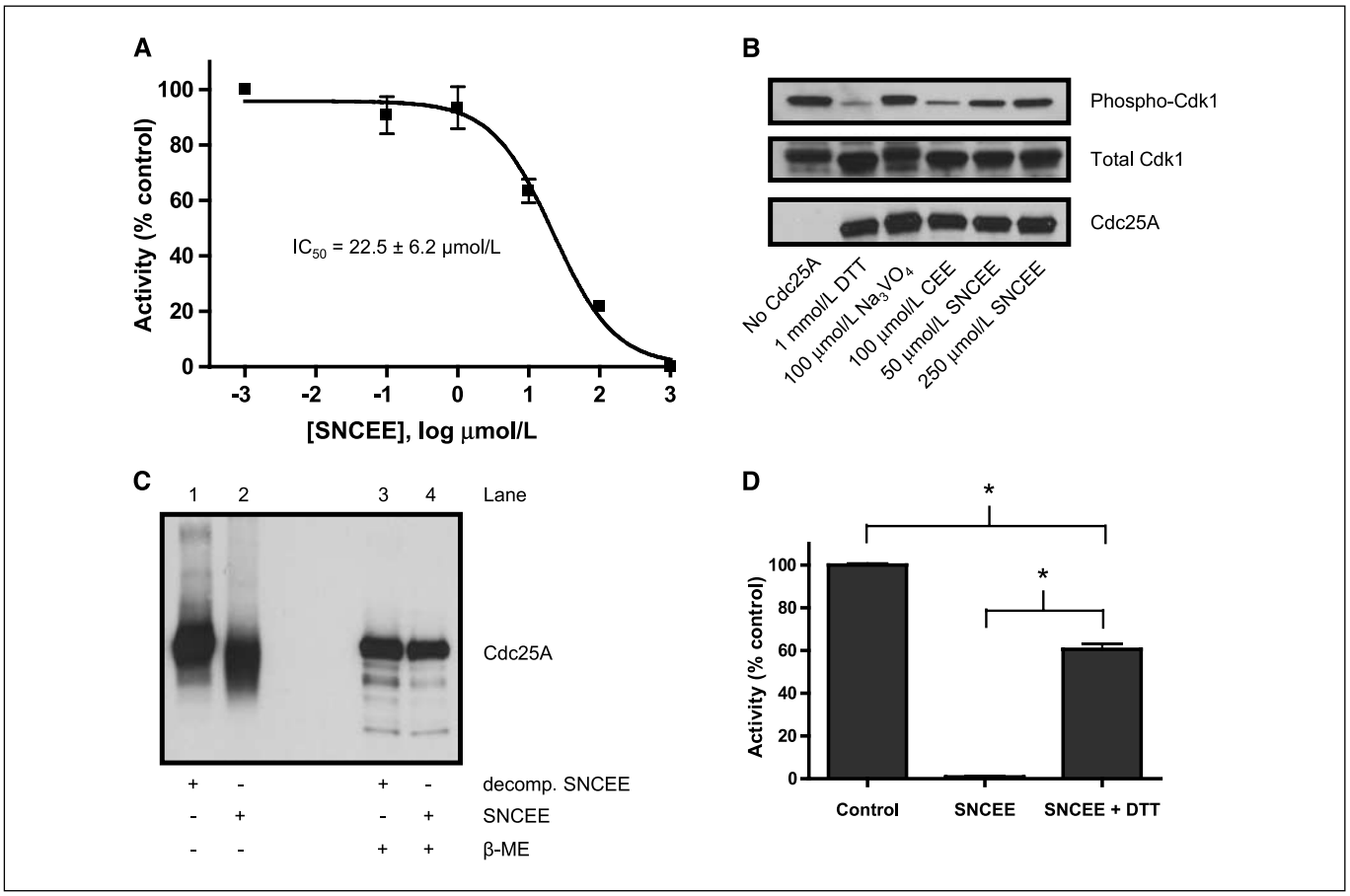

Figure 1. Low molecular mass RSNOs inhibited Cdc25A phosphatase activity in a redox-dependent manner. *A,* rHis-Cdc25A (500 ng) was pretreated for 30 min with the indicated concentrations of SNCEE before assay of OMFP phosphatase activity at 25°C over 1 h. Results are expressed as percentage of vehicle-pretreated Cdc25A activity. *B,* rHis-Cdc25A (500 ng) was pretreated for 30 min with 1 mmol/L DTT, 100 μmol/L Na_3VO_4, or the indicated concentrations of CEE and SNCEE at which time it was incubated with 250 μg cyclin B_1 immunoprecipitate for 60 min at 37°C. Levels of phosphorylated Tyr^{15} Cdk1, total Cdk1, and Cdc25A were then determined by Western blotting. *C,* rHis-Cdc25A was treated with 100 μmol/L decomposed SNCEE or SNCEE and then with β-mercaptoethanol or not before SDS-PAGE. Migration of Cdc25A was then assessed by Western blotting with Cdc25A antibodies. *D,* rHis-Cdc25A was treated with 100 μmol/L SNCEE or not as in Fig. 1*B*, at which time 20 mmol/L DTT was added where indicated immediately before assaying OMFP phosphatase activity. *, $P < 0.001$.

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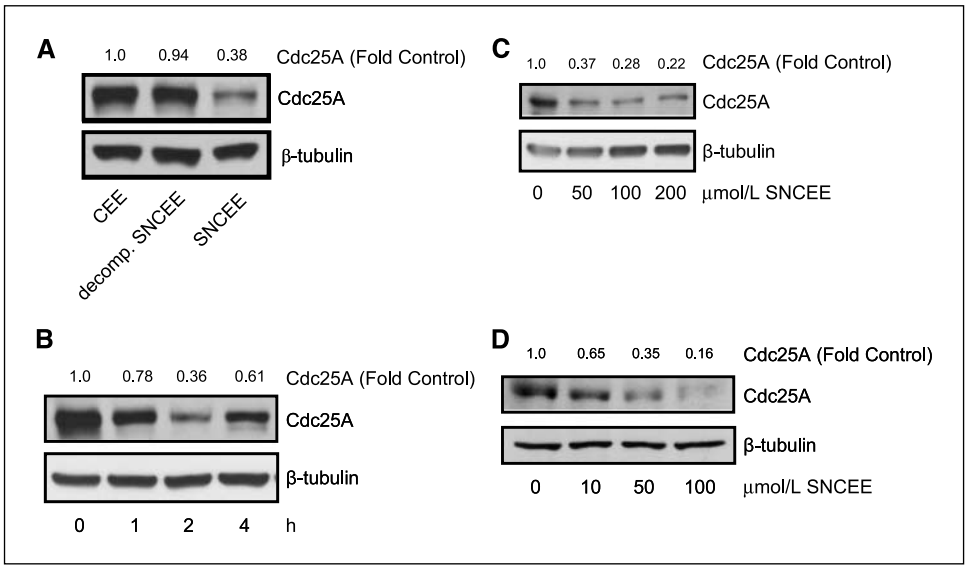

Figure 2. Concentration-dependent and time-dependent decrease in Cdc25A after SNCEE treatment. *A*, HCT116 cells were treated with 100 μmol/L of the indicated compounds and harvested 2 h later for Western blotting. *B*, HCT116 cells were treated with 100 μmol/L SNCEE, and samples were harvested at the indicated time points for Western blotting. *C* and *D,* HCT116 cells (*C*) and HeLa cells (*D*) were treated with the indicated concentrations of SNCEE and harvested 2 h posttreatment for Western blotting. The values above the Western blots are the quantification of band density normalized to β-tubulin from 3-4 independent experiments.

decomposition of SNCEE spectroscopically by the loss of absorbance of the S-N bond at 343 nm before use. All manipulations of cells, lysates and solutions containing RSNOs were performed under subdued lighting.

Adenoviral infection. HCT116 cells in 6-cm dishes were infected with 10 multiplicity of infection (MOI) Ad-LacZ or Ad-iNOS (a gift from Dr. Paul Robbins, University of Pittsburgh) in 1.2 mL PBS in a humidified 37°C incubator for 1 h, after which medium with or without 1 mmol/L L-NMMA was added to the cells for 24 h before harvesting.

Transfection experiments. HCT116 cells were transfected with plasmids encoding HA-tagged Cdc25A, untagged Cdc25A, and HA-tagged ASK-1 using LipofectAMINE PLUS (Invitrogen) in serum-containing medium according to the manufacturer's instructions. Medium containing DNA-lipid complexes was aspirated 3 h after transfection and replaced with complete growth medium. We visually estimated the transfection efficiency at 40% to 50% in cells transfected as above with green fluorescent protein via green fluorescence.

Estimation of nitric oxide production. The nitrate/nitrite colorimetric assay kit (Cayman Chemical) was used according to the manufacturer's instructions to quantify nitrite and nitrate in culture medium as a measurement of •NO production in iNOS-expressing cells 24 h after infection.

Fluorescence microscopy and cytotoxicity assays. Cells were washed once with PBS before addition of 4% formaldehyde in PBS for 10 min at ambient temperature. Cells were washed twice with PBS, and nuclei were stained with 1 μg/mL Hoechst 33342 in PBS. Apoptotic nuclei were counted in three fields of view containing >100 cells each at 20× magnification for each sample. Cell viability was determined using the CellTiter-Blue assay from Promega according to the manufacturer's instructions.

Immunoblotting and coimmunoprecipitation. Cells were harvested in a modified radioimmunoprecipitation buffer (21) and either sonicated as above or incubated on ice for 30 min with frequent vortexing. Lysates were cleared by centrifugation at 13,000 × *g* for 15 min. Protein content was determined by the method of Bradford. Total cell lysates (30–50 μg protein) were resolved by SDS-PAGE using Tris-glycine gels [8% for Cdc25A, Cdc25B, Cdc25C, iNOS, poly (ADP-ribose) polymerase (PARP), HA-ASK-1, and β-tubulin Western blotting and 12% for Cdk1, phosphorylated Tyr^{15} Cdk1, caspase-3, eIF2α, and phosphorylated Ser^{51} eIF2α Western blotting] and transferred to nitrocellulose membranes at 4°C overnight at 35 V for Western blotting. Antibodies against Cdc25A, Cdc25C, Cdk1, and anti–cyclin B_1-agarose were obtained from Santa Cruz Biotechnology. Antibodies against Cdc25B and iNOS/NOS type II were from BD Transduction Laboratories. Antibodies recognizing phosphorylated Tyr^{15} Cdk1, phosphorylated Ser^{51} eIF2α, eIF2α, and PARP were from Cell Signaling Technology, the β-tubulin antibody was from Cedarlane Laboratories, the caspase-3 antibody was from Assay Designs, and the HA antibody was from Covance. 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). Films were scanned using an Amersham Biosciences SI densitometer and analyzed using ImageQuant software (Amersham Biosciences) for quantification. For coimmunoprecipitation, cells were harvested in HEPES lysis buffer (5), and 1.5 mg of protein were precleared with 2 μg of normal mouse IgG followed by incubation with 50 μL of HA.11-affinity matrix (Covance) overnight at 4°C. After washing with HEPES lysis buffer, bound proteins were eluted into Laemmli buffer and subjected to SDS-PAGE as described above.

Production of rHis-Cdc25A and Tyr15-hyperphosphorylated Cdk1/cyclin B1 and Cdc25A phosphatase assays. rHis-Cdc25A was produced in *Escherichia coli* and purified using nickel-nitrilotriacetic acid (His_6) resin, as described previously (22), except that the protein was eluted in the absence of reducing agents. To generate phosphorylated Tyr^{15} Cdk1, we treated subconfluent HeLa cells for 1 h with 40 μmol/L etoposide, and 23 h later, cells were lysed as above. Cyclin B_1–associated Cdk1 was coimmunoprecipitated using an agarose-conjugated anti–cyclin B_1 antibody. Precipitated protein was frozen at −80°C until use. Dephosphorylation of Cdk1 was measured by incubating 500 ng of rHis-Cdc25A with 250 μg of cyclin B_1 immunoprecipitate in a 50-μL final volume for 60 min at 37°C. Loading buffer was then added, and samples were boiled to halt the reaction. Cdk1 phosphorylation at Tyr^{15} was determined by Western blotting as above using a phosphorylated Tyr^{15} Cdk1 antibody. Cdc25A phosphatase activity was measured at pH 7.4 and at ambient temperature with the artificial substrate *O*-methylfluorescein phosphate (OMFP) at its K_m in a 96-well microtiter plate assay based on previously described methods (22). Fluorescence emission (ex 485 nm, em 525 nm) was measured after a 60-min incubation period with a Molecular Devices Corp. M5 Spectrophotometer.

UV treatment. Cells were washed once with PBS before UV irradiation (UVC Crosslinker, Stratagene), followed by addition of fresh medium before incubation for the indicated times and cell harvesting.

Radioisotope incorporation studies. Cells were washed twice with PBS and incubated with priming medium (DMEM lacking L-cysteine or L-methionine, supplemented with 10% dialyzed FBS, 100 units/mL penicillin/streptomycin, and 2 mmol/L L-glutamine) for 1 h before addition of 300 μCi/mL of EasyTag EXPRESS [^{35}S] Protein Labeling Mix (Perkin-Elmer).

Statistical analysis. Results were expressed as means ± SE of at least three independent experiments. ANOVA and *t* tests were performed using

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Graphpad Prism 4 software (Graphpad Software). Differences were considered statistically different if $P < 0.05$. Western blots and autoradiograms were representative of at least three independent experiments.

Results

***S*-nitrosothiols inhibited Cdc25A phosphatase activity.** The Cdc25A catalytic cysteine is predicted to have a low pK_a and exist primarily as a highly reactive thiolate anion (8, 23). The Cdc25A catalytic domain crystal structure indicates that this catalytic thiolate exists in a hydrophobic pocket and lies in an acid-base motif (24, 25). As this catalytic thiolate is essential for its enzymatic activity, *S*-nitrosation of the Cdc25A catalytic cysteine would be expected to render the enzyme inactive. Because nitrosating agents appear to preferentially *S*-nitrosate thiolates that exist in acid-base motifs and partition selectively in hydrophobic environments (16, 17), we tested whether low molecular mass RSNOs regulated Cdc25A phosphatase activity. We incubated recombinant His-tagged Cdc25A with the low molecular mass RSNO SNCEE and measured Cdc25A phosphatase activity with the artificial substrate OMFP. As shown in Fig. 1*A*, SNCEE inhibited the activity of Cdc25A with an IC_{50} value of 22.5 $\pm$ 6.2 μmol/L. Inhibition of Cdc25A by SNCEE was mediated by the intact *S*-nitrosothiol as SNCEE was stable over the course of the assay and decomposition of SNCEE before treatment of Cdc25A compromised its activity (Supplementary Fig. S1). We also queried the effects of SNCEE on Cdc25A activity toward its endogenous substrate, Cdk1/cyclin B complex. As illustrated in Fig. 1*B*, dephosphorylation of $Cdk1^{Tyr15}$ required Cdc25A. Treatment of Cdc25A with SNCEE before incubation with phosphorylated Tyr^{15} Cdk1/cyclin B prevented dephosphorylation of $Cdk1^{Tyr15}$. SNCEE did not inhibit Cdk2 activity *in vitro*, suggesting some specificity for Cdc25A (data not shown). Together, these results indicate that low molecular mass RSNOs regulate the phosphatase activity of Cdc25A.

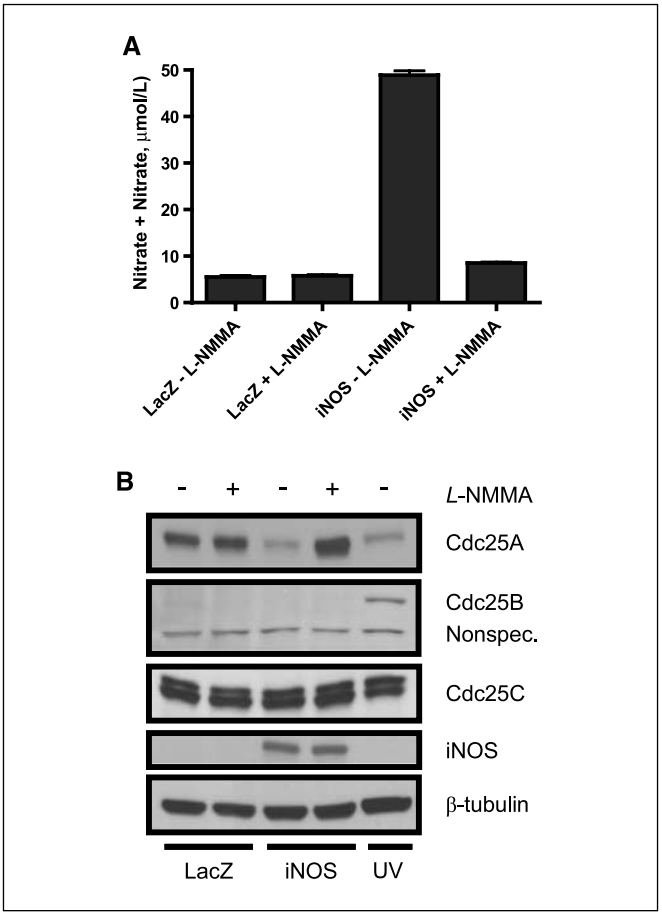

Figure 3. Regulation of Cdc25A expression by iNOS. *A,* the concentration of nitrite and nitrate in the medium (from *B*) was determined using a colorimetric detection kit from Cayman Chemical according to the manufacturer's instructions. *B,* HCT116 cells were infected with 10 MOI of adenoviruses encoding the *β-galactosidase* gene (LacZ) or the human *iNOS* cDNA in the presence or absence of 1 mmol/L L-NMMA. 24 h later, cells were harvested for Western blotting.

SNCEE induced redox-sensitive changes in Cdc25A. Upon reaction with protein thiols RSNOs can generate *S*-nitrosothiols, protein disulfides, and mixed disulfides (26). Each of these is reversible with reductants. Thus, we examined whether SNCEE caused reductant-sensitive changes in Cdc25A. We treated Cdc25A with SNCEE or decomposed SNCEE and monitored its electrophoretic mobility under reducing and nonreducing conditions (Fig. 1*C*). SNCEE-treated Cdc25A migrated more rapidly than decomposed SNCEE-treated Cdc25A under nonreducing conditions (Fig. 1*C, lane 1* versus *lane 2*); addition of β-mercaptoethanol to Cdc25A before SDS-PAGE ablated this enhanced migration (Fig. 1*C, lanes 3* and *4*). Vicinal dithiols, such as thioredoxin and DTT, denitrosate protein RSNOs (27). To investigate the potential biochemical significance of this change in mobility of Cdc25A, we treated Cdc25A with SNCEE or decomposed SNCEE and added DTT coincident with OMFP before measuring Cdc25A phosphatase activity. Figure 1*D* shows that preaddition of DTT before the phosphatase assay restored the majority of Cdc25A activity attenuated by SNCEE. Full activity may not have been restored due to the time dependence of DTT action. Together, these results were consistent with a model in which a redox-sensitive modification to the Cdc25A protein structure induced by low molecular mass RSNOs inhibited Cdc25A phosphatase activity.

Cdc25A protein levels were decreased after SNCEE treatment in multiple tumor cell lines. We next investigated the effects of nitrosative insult on cellular Cdc25A using SNCEE. We treated HCT116 cells with 100 μmol/L SNCEE or the control compounds L-cysteine ethyl ester (CEE) or decomposed SNCEE, as 100 μmol/L SNCEE induced significant accumulation of intracellular RSNOs, but did not produce accumulation of the oxidative and nitrative stress marker 3-nitrotyrosine, which decreased moderately in some but not all experiments (Supplementary Fig. S2). Surprisingly, SNCEE, but neither decomposed SNCEE nor CEE, decreased Cdc25A protein levels; the protein levels of β-tubulin were unaffected (Fig. 2*A*). We observed no significant change in the protein levels of Cdk2 or glyceraldehyde-3-phosphate dehydrogenase (Supplementary Fig. S3), indicating some specificity for Cdc25A. Cdc25A loss was time-dependent, with the lowest Cdc25A levels occurring ~2 h after treatment and rebounding by 4 h posttreatment (Fig. 2*B*). Cdc25A suppression after SNCEE was concentration-dependent; treatment of HCT116 cells with 50 μmol/L SNCEE resulted in loss of >60% of Cdc25A by 2 h after treatment (Fig. 2*C*). A similar concentration-dependent loss of Cdc25A protein levels in response to SNCEE was observed in HeLa cervical carcinoma cells (Fig. 2*D*) indicating that SNCEE decreased Cdc25A levels in cells derived from multiple tumor types. Recognition of Cdc25A by the antibody used for Western blotting was unaffected by RSNOs directly under the reducing conditions of SDS-PAGE (Fig. 1*C*), suggesting a *bona fide* decrease in Cdc25A protein levels.

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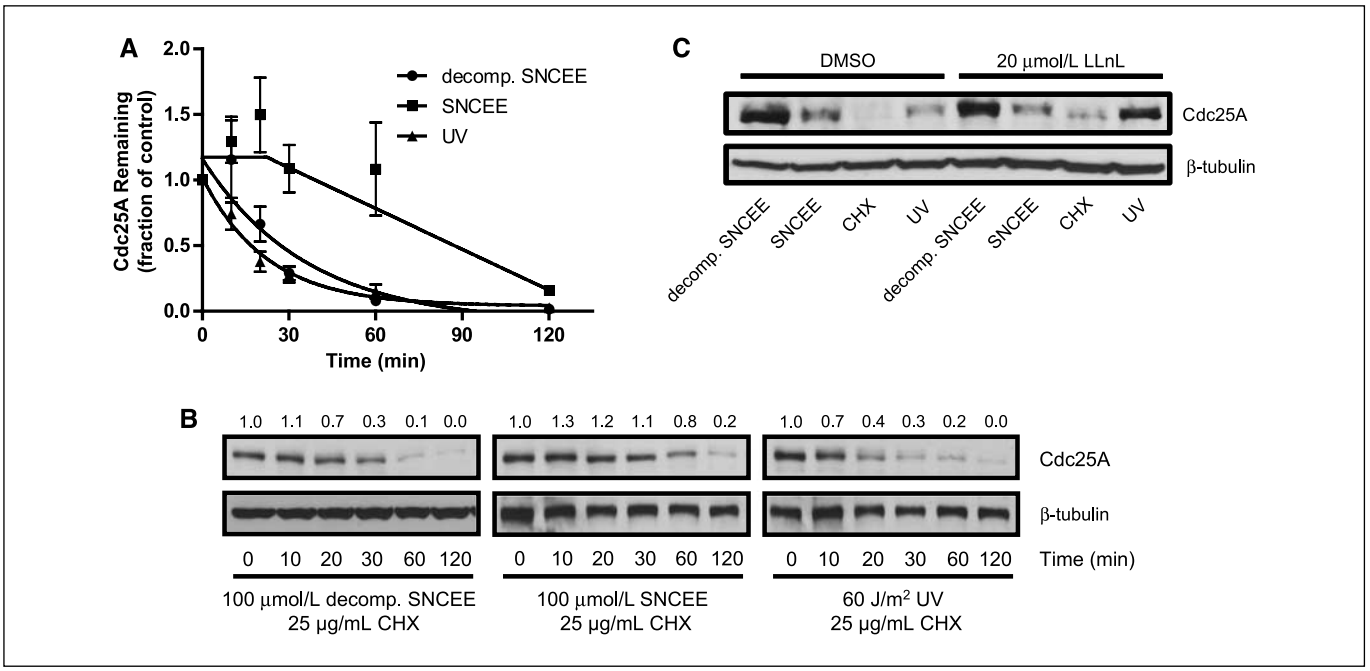

Figure 4. Cdc25A protein stability was not decreased after SNCEE treatment. *A,* HCT116 cells were cotreated with 25 μg/mL CHX and either 100 μmol/L decomposed SNCEE, 100 μmol/L SNCEE, or 60 J/m^2 UV. Cells were harvested at the indicated time points for Western blotting. Western blots were then densitometrically scanned, and remaining Cdc25A levels were expressed as fraction Cdc25A at time = 0 after normalization to β-tubulin. *B*, representative Western blots used to generate *A*. *C,* HCT116 cells were treated simultaneously with DMSO or 20 μmol/L LLnL and with 100 μmol/L of the indicated compounds or 60 J/m^2 UV irradiation as described in Materials and Methods. Cells were harvested 2 h later for Western blotting.

iNOS-derived •NO decreased Cdc25A expression. In cells iNOS catalyzes RSNO production and initiates nitrosative stress (15). To investigate whether intracellular production of •NO from an endogenous source affected Cdc25A expression, we infected HCT116 cells with adenovirus encoding the human *iNOS* cDNA. Expression of iNOS induced •NO formation (Fig. 3*A*) similar to the concentrations of SNCEE used above and did not induce nitration (Supplementary Fig. S4). Production of •NO from iNOS decreased Cdc25A protein levels but did not affect Cdc25B or Cdc25C (Fig. 3*B*). Loss of Cdc25A was independent of multiple known regulators of Cdc25A stability or transcription (Supplementary Fig. S5). Blockade of •NO production by the NOS inhibitor L-NMMA prevented •NO generation and restored Cdc25A levels (Fig. 3*A* and *B*). These results show that endogenously generated •NO decreased Cdc25A protein levels.

Cdc25A half-life was not shortened after SNCEE treatment. Cdc25A is a labile protein with a short half-life (1). In response to various stresses, Cdc25A becomes hyperphosphorylated by several stress-dependent kinases, including p38, Chk1, and Chk2, subsequently targeting Cdc25A for degradation via ubiquitin-mediated proteolysis (1). To determine whether nitrosative stress suppressed Cdc25A by decreasing its protein half-life, we treated HCT116 cells with UV irradiation, which decreases Cdc25A half-life (28), or either decomposed SNCEE or SNCEE, and monitored its half-life after blockade of new protein synthesis with CHX (Fig. 4*A* and *B*). Logarithmic regression analysis of the data indicated half-lives of 25.1 and 15.5 min for Cdc25A in decomposed SNCEE-treated and UV-treated cells, respectively, consistent with UV-induced accelerated Cdc25A turnover. Cdc25A did not decrease logarithmically after SNCEE treatment; rather, Cdc25A levels seemed to be temporarily stabilized, although by 120 min most of the Cdc25A was lost (Fig. 4*A* and *B*). We hypothesize this may be due to inhibition of one or more Cdc25A E1 or E2 ubiquitin ligases, which also contain catalytic cysteines. These results suggested that Cdc25A stability was not decreased in response to SNCEE treatment. Similarly, pretreatment of HCT116 cells with the proteasomal inhibitor LLnL blocked UV-induced Cdc25A loss, but not Cdc25A loss due to treatment with SNCEE or the protein synthesis inhibitor CHX (Fig. 4*C*), distinguishing these from modulators of Cdc25A protein turnover. Collectively, these results argued against a proteasomal mechanism of Cdc25A loss in response to SNCEE.

SNCEE repressed protein translation and down-regulated Cdc25A posttranscriptionally. Transcription from the Cdc25A promoter is negatively regulated by several known stress-responsive proteins, including p53, p21, and HIF-1α (29–31). •NO or nitrosative stress have been reported to activate and/or stabilize the expression of several of these proteins (32–34); thus, we investigated whether the Cdc25A promoter region was essential for SNCEE-mediated Cdc25A suppression. We transfected HCT116 cells with vectors containing the *CDC25A* cDNA under the control of the cytomegalovirus promoter and monitored the effect of SNCEE on Cdc25A levels. Decreased HA-Cdc25A levels were observed with SNCEE but not decomposed SNCEE or CEE (Fig. 5*A*). This implied that down-regulation of Cdc25A after SNCEE was a promoter-independent, posttranscriptional effect.

Attenuation of protein translation provides a functional mechanism to decrease the protein levels of a rapidly synthesized protein. The short half-life of Cdc25A indicated a rapid synthetic rate for Cdc25A, so we investigated whether SNCEE affected overall protein synthesis. We treated HCT116 cells with radiolabeled [^{35}S]cysteine and [^{35}S]methionine for different time periods after

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decomposed SNCEE or SNCEE treatment and monitored total radioisotope incorporation into proteins via SDS-PAGE and autoradiography (Fig. 5*B*). Total protein loading was equal as assessed by β-tubulin levels; radiolabeled amino acid incorporation into protein from SNCEE-treated cell lysates, however, was reduced during both the first and second hours, consistent with the time course of Cdc25A decrease. In addition, the magnitude of protein synthesis repression was consistent with the expression levels of Cdc25A in SNCEE-treated cells. Together, these results suggested that SNCEE treatment decreased Cdc25A protein translation.

eIF2α regulated basal Cdc25A levels and response to SNCEE. SNCEE decreased global protein synthesis. Stress-dependent global translational inhibition is mediated primarily through phosphorylation and inhibition of the translational regulator eIF2α (35). In response to various stresses, eIF2α is phosphorylated on Ser^{51} by stress-sensitive kinases (36). Phosphorylation of eIF2α at Ser^{51} increases its affinity for the eIF2B subunit, whose release from the eIF2 complex is necessary for GDP-GTP recycling and subsequent tRNA recruitment and binding (35). Thus, $eIF2\alpha^{Ser51}$ hyperphosphorylation results in a general decrease in protein translation. To investigate whether SNCEE altered the activity of eIF2α, we treated cells with 100 μmol/L SNCEE and monitored phosphorylation of $eIF2\alpha^{Ser51}$ using phosphorylated-specific antibodies (Fig. 5*C*). Although the total levels of eIF2α were not changed in response to SNCEE, the pool of phosphorylated $eIF2\alpha^{Ser51}$ increased in a time-dependent manner with phosphorylated $eIF2\alpha^{Ser51}$ appearing as soon as 30 minutes after SNCEE treatment and persisting for at least 2 hours after treatment. The kinetics of eIF2α hyperphosphorylation were consistent with the loss of Cdc25A protein, as well as with attenuation of protein synthesis in response to SNCEE.

To determine whether inhibition of eIF2α was sufficient to suppress Cdc25A protein levels, we treated HCT116 cells with the eIF2α inhibitor salubrinal (37) or vehicle and determined the effects on Cdc25A expression by Western blotting (Fig. 5*D*). In response to eIF2α inhibition, Cdc25A levels decreased to levels similar to those observed in SNCEE-treated cells. This implied that eIF2α was a regulator of basal Cdc25A protein levels and suggested that eIF2α inhibition in response to SNCEE was the mechanism by which nitrosative stress decreased Cdc25A.

Nitrosative stress decreased the fraction of Cdc25A-bound ASK-1 and sensitized cancer cells to chemotherapeutic-induced apoptosis. Cdc25A protects against apoptosis by binding to and inhibiting ASK-1 (5). We hypothesized that Cdc25A protein modification (Fig. 1*C*) or suppression (Fig. 2*A*) by nitrosative stress would sensitize cells to apoptotic stimuli by decreasing its association with ASK-1. We expressed Cdc25A and HA-tagged ASK-1 (HA-ASK-1) in HCT116 cells and measured the effect of SNCEE-induced nitrosative stress on Cdc25A-ASK-1 interaction by coimmunoprecipitation (Fig. 6*A*). SNCEE decreased the amount of Cdc25A associated with ASK-1 at 2 hours, consistent with Cdc25A loss after SNCEE (Fig. 2*B*).

CDDP induces apoptosis through the ASK-1 pathway (38). We exposed cells to 10 μmol/L CDDP or vehicle after pretreatment with 100 μmol/L of either decomposed SNCEE or fresh SNCEE and measured apoptosis after 24 and 48 hours (Fig. 6*B* and *C*). SNCEE alone did not affect basal nuclear fragmentation frequency compared with decomposed SNCEE (2.08 ± 0.51% versus 1.58 ± 0.31%; Fig. 6*B*), and pretreatment of cells with SNCEE increased apoptosis 2-fold (13.83 ± 1.37%) after CDDP compared with decomposed SNCEE-pretreated cells (7.00 ± 0.87%). Similarly, SNCEE pretreatment before 10 μmol/L CDDP induced cleavage of PARP and procaspase-3 as evidenced by accumulation of cleaved PARP and p17/p20 caspase-3 subunits (Fig. 6*C*) whereas decomposed SNCEE and CDDP cotreatment did not. In agreement with sensitization to CDDP-induced apoptosis by SNCEE, cell viability

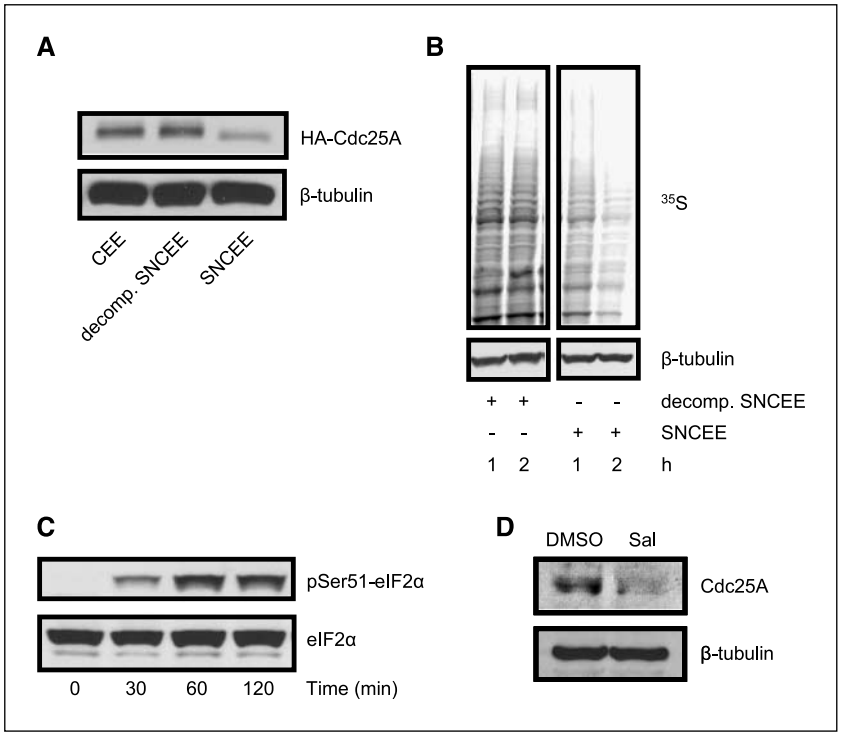

Figure 5. eIF2α-mediated translational Cdc25A suppression after SNCEE treatment. *A,* HCT116 cells were transfected with plasmids encoding HA-tagged Cdc25A as described in Materials and Methods. After 24 h, cells were treated with 100 μmol/L of the indicated compounds. 2 h later, cells were harvested for Western blotting. *B,* HCT116 cells were incubated for 1 h in medium lacking L-Cys and L-Met and then treated with 100 μmol/L decomposed SNCEE or 100 μmol/L SNCEE for 2 h. We added 300 μCi/mL [^{35}S]L-Cys and [^{35}S]L-Met to the medium at the start of the indicated hour post-SNCEE treatment, and cells were harvested for autoradiography and Western blotting 60 min later. Nonadjacent lanes are shown from the same gel. *C,* HCT116 cells were treated for the indicated times with 100 μmol/L SNCEE and harvested for Western blotting. *D,* HCT116 cells were treated for 24 h with DMSO or with 75 μmol/L salubrinal (*Sal*) and were then harvested for Western blotting.

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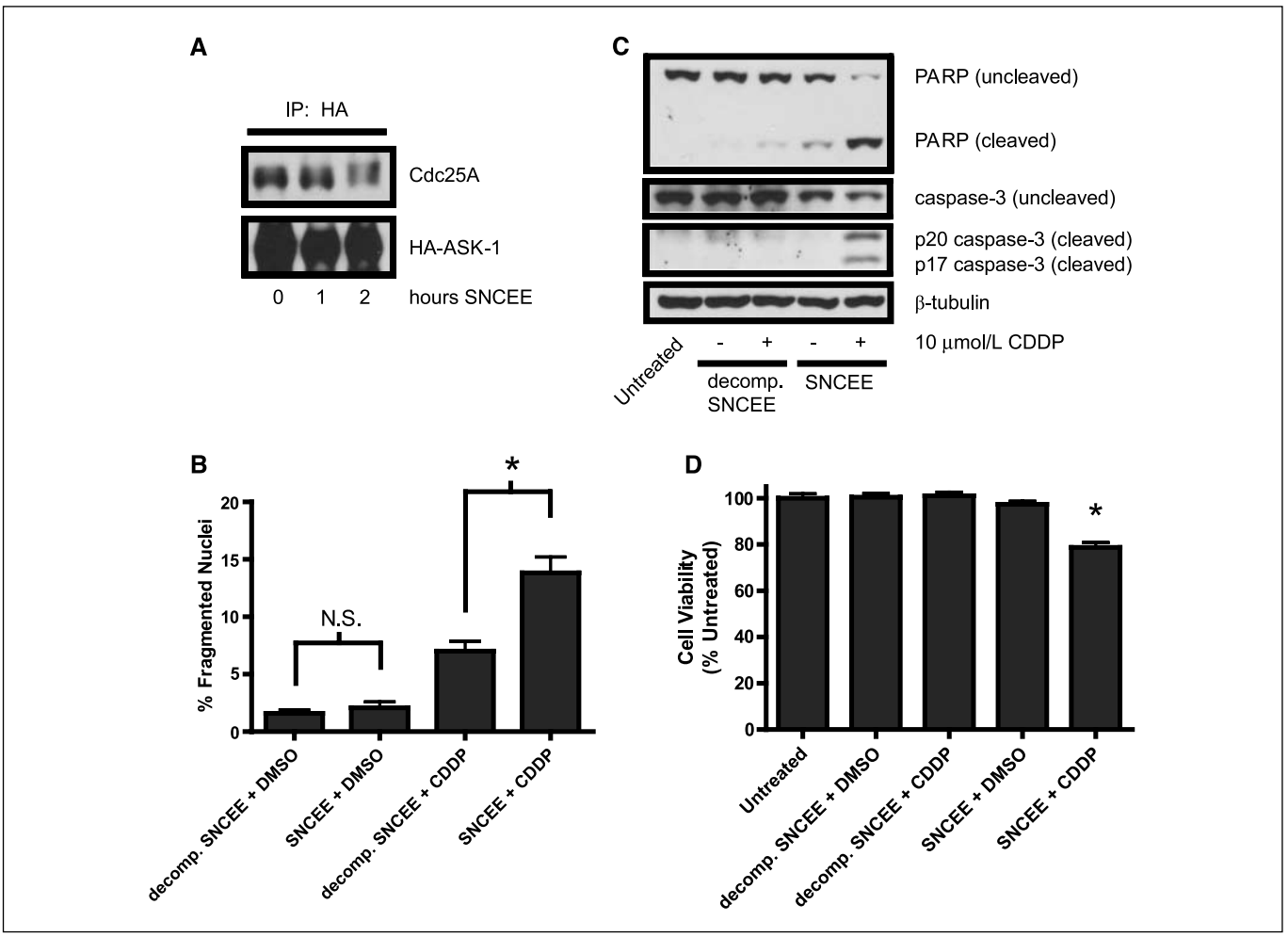

Figure 6. Nitrosative stress attenuated Cdc25A binding to ASK-1 and sensitized cells to apoptosis. *A,* HCT116 cells expressing Cdc25A and HA-ASK-1 were treated for the indicated times with 100 μmol/L SNCEE. Cells were harvested, and HA-ASK-1 was immunoprecipitated as described in Materials and Methods. Immunoprecipitates were subjected to Western blotting with the indicated antibodies. *B,* HCT116 cells were pretreated with 100 μmol/L decomposed SNCEE or fresh SNCEE for 1 h before exposure to 10 μmol/L CDDP. At 48 h after CDDP treatment, cells were fixed, nuclei were stained, and apoptotic nuclei were counted. *N.S.*, not significant; *, $P < 0.0001$. *C,* HCT116 cells were treated as described in *B* and harvested after 24 h for Western blotting with the indicated antibodies. *D,* HCT116 cells were treated as described in *B*, and cell viability was measured 24 h later using the CellTiter Blue assay. *, $P < 0.0001$.

was decreased by >20% in SNCEE and CDDP cotreated cells, whereas neither SNCEE alone nor decomposed SNCEE and CDDP cotreatment significantly affected cell viability (Fig. 6*D*). Together, these results indicate that nitrosative stress decreased Cdc25A association with ASK-1 and sensitized cells to apoptotic cell death, consistent with an inhibitory role for Cdc25A in ASK-1–mediated apoptosis.

Discussion

Stringent Cdc25A regulation is critical for cell growth without unwarranted proliferation. High Cdc25A expression and activity are hallmarks of human cancers, likely conveying resistance to apoptosis and to antigrowth signals. Thus, mechanisms have evolved to rapidly suppress Cdc25A after normal and stress-mediated cellular signaling. Previous research uncovered transcriptional and proteasomal control of Cdc25A after stress; herein, we report two distinct mechanisms regulating Cdc25A after exposure to •NO and RNS: translational suppression after nitrosative stress and enzymatic inhibition of Cdc25A by low molecular mass RSNOs. These mechanisms may be most prevalent in tumor tissues expressing iNOS or in tumors derived from chronic inflammatory diseases, as •NO generated from iNOS was sufficient to suppress Cdc25A levels (Fig. 3*B*).

SNCEE reversibly inhibited Cdc25A activity. RSNOs can induce *S*-nitrosation of target cysteines and generate mixed disulfides from thiols (26, 39). These modifications to Cdc25A could induce a migration shift by SDS-PAGE, would be reversible with DTT, and would be expected to inhibit its phosphatase activity if the catalytic cysteine were modified. Alternatively, generation of an intramolecular disulfide bond between the active site thiolate and a proximal thiol, as reported for H_2O_2-treated Cdc25, could occur (8). Our preliminary experiments indicate that 100 μmol/L SNCEE induced *S*-nitrosation of 2 mol cysteine/mol Cdc25A (data not shown), and we are currently using mass spectrometry to identify the specific sites of *S*-nitrosation in Cdc25A.

Translational Cdc25A suppression after nitrosative stress can be distinguished from previous reports examining Cdc25A regulation by RNS (11). In response to nitrating agents, Cdc25A loss was paralleled by activation of the upstream kinase ATM and was

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sensitive to okadaic acid. Protein phosphatase PP5 activity is required for ATM activity (40), and PP5 is inhibited by okadaic acid (41). These data imply the traditional DNA damage pathway mediates Cdc25A loss after $\bullet NO_2$ or SIN-1 treatment. In contrast, SNCEE did not decrease Cdc25A half-life nor was Cdc25A loss blocked by proteasome inhibition. Also, pretreatment with the ATM/ATR inhibitor caffeine did not block Cdc25A loss after SNCEE treatment, although UV-induced Cdc25A loss was inhibited (Supplementary Fig. S6). This further distinguished SNCEE-mediated Cdc25A down-regulation from the traditional DNA damage pathway. Collectively, this work and previous studies (11) reinforce the concept that distinct RNS mediate discrete intracellular signaling.

$eIF2\alpha^{Ser51}$ is phosphorylated by the stress-responsive eIF2 kinases PKR-like endoplasmic reticulum kinase (PERK), heme-regulated inhibitor (HRI), GCN2, and RNA-dependent protein kinase (36) and dephosphorylated by protein phosphatase 1 (PP1; refs. 37, 42). How eIF2α becomes hyperphosphorylated in response to RNS is unknown, although several candidate mediators exist. PP1 is inhibited by H_2O_2 in PC12 cells and suppression of PP1 activity in H_2O_2-treated cells correlated with phosphorylation of $eIF2\alpha^{Ser51}$ (43). H_2O_2 can deplete thiols by oxidation to intermolecular and intramolecular disulfides or higher-order cysteine oxides. SNCEE depleted thiols (Supplementary Fig. S2*B*), indicating that this could be responsible for $eIF2\alpha^{Ser51}$ hyperphosphorylation in response to RSNOs.

Perturbations to the ER redox status either in response to reductants (44) or RNS are reported to initiate ER stress and, thus, generate phosphorylated $eIF2\alpha^{Ser51}$, presumably through activation of PERK (45). Whereas •NO-derived species have not been reported to directly activate PERK, *S*-nitrosation of the ER-localized protein disulfide isomerase results in protein misfolding, which is a well-characterized ER stress (46). This could initiate eIF2α hyperphosphorylation and subsequent translational inhibition. Although PERK-mediated translational inhibition can occur rapidly in response to several stimuli (44), it remains undetermined whether PERK is activated in response to RNS, or whether *S*-nitrosation of protein disulfide isomerase and subsequent ER stress is mediated rapidly enough to elicit •NO-induced and SNCEE-induced loss of Cdc25A.

In addition to PERK activation, HRI kinase activity has previously been reported to be activated in response to •NO. HRI may not be the major target of SNCEE-induced eIF2α hyperphosphorylation in HCT116 cells, as HRI protein is expressed primarily in erythroid precursor cells, and HRI is essentially undetectable in many other cell types (47). Nonetheless, it remains possible that HRI mediated eIF2α activation.

Cdc25A inhibits apoptosis by binding to and inhibiting the proapoptotic mitogen-activated protein kinase family member ASK-1 (5). Overexpression of Cdc25A attenuates ASK-1 activation and apoptosis in response to H_2O_2, suggesting that dissociation of Cdc25A from ASK-1 is a required step for stimulation of ASK-1 kinase activity (5). Nitrosative stress decreased the Cdc25A-bound fraction of ASK-1 (Fig. 6*A*). Apoptotic death induced by CDDP is ASK-1–dependent (38) and was increased in cells pretreated with SNCEE. The decoupling of Cdc25A from ASK-1 may be a prerequisite for ASK-1–dependent apoptosis; thus translational suppression of Cdc25A after nitrosative stress may represent cellular priming of the apoptotic machinery. We have observed activation of the ASK-1 downstream target kinase p38 after SNCEE treatment with kinetics similar to loss of Cdc25A,[1] consistent with a model where suppression of Cdc25A after stress generated by high •NO primes the cell for ASK-1 activation and apoptotic signaling through the p38 pathway. Future studies in our laboratory are centered on testing this hypothesis.

In summary, we have described novel regulation of Cdc25A in response to •NO and •NO-derived species: RSNOs reversibly inhibit Cdc25A phosphatase activity, whereas inhibition of eIF2α after nitrosative stress suppresses translation of Cdc25A protein. SNCEE attenuated inhibitory binding of Cdc25A to ASK-1 and sensitized cells to apoptosis. Together, these results highlight the importance of stringent control of Cdc25A to regulate cellular activities. We speculate that this multifaceted control of Cdc25A allows a cellular "stopwatch" function, where rapid inhibition of Cdc25A phosphatase activity upon RSNO accumulation blunts phosphatase activity-dependent Cdc25A signaling, whereas prolonged or severe •NO-mediated cell stress suppresses Cdc25A levels and attenuates nonenzymatic Cdc25A functions, such as apoptosis suppression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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