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Human Ogg1, a Protein Involved in the Repair of 8-Oxoguanine, Is Inhibited by Nitric Oxide[1]

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ABSTRACT

NO-mediated inhibition of base excision DNA repair may potentiate oxidative DNA damage in cells and could be relevant to carcinogenesis associated with chronic inflammation. Because 8-oxoguanine, a ubiquitous oxidative DNA lesion, is repaired predominantly by human 8-oxoguanine glycosylase (hOgg1), our aim was to determine whether NO directly inhibits its repair activity. Neither induction of NO-generating enzyme inducible NO synthase nor treatment with *S*-nitroso-*N*-acetyl-D-L-pencillamine altered expression of hOgg1 in a human cholangiocarcinoma cell line (KMBC). In contrast, both treatments completely inhibited activity of hOgg1 immunoprecipitated from KMBC cells overexpressing hOgg1 and in a cell-free system. Both NO and peroxynitrite were capable of inhibiting hOgg1 activity. Inhibition of hOgg1 protein was characterized by formation of *S*-nitrosothiol adducts and loss/ejection of zinc ions. Our data indicate that NO, an inflammatory mediator, directly inhibits a key base excision repair enzyme (hOgg1) responsible for base excision repair of 8-oxoguanine. These data support the concept that NO-mediated inhibition of DNA contributes to the mutagenic environment of chronic inflammation.

INTRODUCTION

Chronic inflammation of gastrointestinal mucosa predisposes individuals to the development of carcinoma in the affected tissues. For example, chronic inflammation of the esophagus, gastric mucosa, pancreas, colon, and liver is a well-established risk factor for malignant transformation in these organs (1–3). Although the cellular mechanisms culminating in malignant transformation of epithelial cells are complex and multifactorial, there is increasing evidence implicating NO as a link between inflammation and carcinogenesis (4). First, chronic inflammation is associated with induction of iNOS,[3] and this enzyme efficiently generates NO (5). Second, NO has been suggested to promote cancer development by acting as an endogenous mutagen, an angiogenesis factor, a mitogen, an enhancer of proto-oncogene expression (6), and an inhibitor of apoptosis (7, 8). Finally, many advanced cancers continue to express iNOS, which suggests that NO contributes to tumor progression (9, 10). Collectively, these data are consistent with a key role for NO in the initiation, promotion, and progression of many human gastrointestinal cancers.

We have been interested in how NO functions as a mutagen using biliary epithelial cells (cholangiocytes) and cholangiocarcinoma cell lines as model systems (11, 12). Our studies have demonstrated that iNOS is not expressed in normal cholangiocytes but is expressed in cholangiocytes of patients with chronic biliary tract inflammation such as primary sclerosing cholangitis, a disease known to predispose individuals to the development of cholangiocarcinoma (11). The expression of iNOS in cholangiocytes is also associated with accumulation of 8-oxodG lesions; 8-oxodG lesion is a dominant oxidative DNA lesion that is highly mutagenic because it predisposes to the development of GC→TA transversions (13, 14). Because 8-oxodG is efficiently repaired in humans by the BER pathway (15, 16), the accumulation of this oxidative DNA lesion in iNOS-expressing cholangiocytes suggests an inhibition of DNA repair. Indeed, we have shown inhibition of both global and base excision DNA repair in cholangiocytes and cholangiocarcinoma cell lines by a NO-dependent mechanism (12). The inhibition of DNA repair by NO could be blocked by NO scavengers but not by inhibitors of the NO-mediated, cGMP-dependent signal transduction pathway (12). These observations suggest that NO may directly inhibit DNA repair enzymes including those responsible for BER. Given the importance of the BER pathway in preventing mutagenesis after oxidative DNA damage, more information is needed with regard to how NO disrupts this pivotal antimutagenesis safeguard.

Oxidative DNA damage is predominantly repaired by BER enzymes. The predominant oxidative mutagenic lesion 8-oxodG in humans is removed by a BER glycosylase termed hOgg1 that is ubiquitously expressed and maps to chromosome 3p26.2 (15, 16). Studies from mice deficient in the gene for murine Ogg1 demonstrate that this enzyme is responsible for >95% of BER activity in mammalian cells (17). Two isoforms of hOgg1 are expressed due to alternative splicing of a single mRNA gene product. The hOgg1-1a protein is localized to the nucleus, and the hOgg1-2a is localized to the mitochondria (18). hOgg1 contains critical thiol moieties that are necessary for catalytic activity (19). The hOgg1 protein has two distinct DNA-binding motifs, a helix-hairpin-helix motif and a C_2H_2 zinc finger motif (15). Like other zinc finger proteins (20), hOgg1 is potentially susceptible to nitrosylation of its thiol moieties (21, 22). Thiol nitrosylation can disrupt the zinc finger motif, resulting in loss of the zinc ion and irreversible disruption of catalytic activity (23).

Thus, the objective of the current study was to determine whether hOgg1-1a is a direct target for nitrosylation with resultant loss of catalytic activity. We used a cholangiocarcinoma cell line for these studies as a model system because the biliary epithelium is a target of chronic inflammation in a variety of cholangiopathies that predispose individuals to the development of cancer. We demonstrate that cellular hOgg1-1a-mediated BER activity is inhibited during exposure to NO. This inhibition was associated with protein nitrosylation and ejection of zinc. These data suggest that NO generated during human inflammatory diseases not only causes oxidative DNA damage but also prevents DNA repair by directly inhibiting key DNA repair enzymes. The inability to repair oxidative DNA lesions would be predicted to be mutagenic and increase the risk of carcinogenesis.

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[3] The abbreviations used are: iNOS, inducible nitric oxide synthase; 8-oxodG, 8-oxoguanine; BER, base excision repair; HA, hemagglutinin; IP, immunoprecipitation; MnTBAP, Mn(III)tetrakis(4-benzoic acid) porphyrin; SNAP, *S*-nitroso-*N*-acetyl-D-L-pencillamine; SIN-1, 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride; SNOC, *S*-nitrosocysteine; WCE, whole cell extract; hOgg1, human 8-oxoguanine glycosylase; rhIL-1β, recombinant human interleukin 1β; rhTNF-α, recombinant human tumor necrosis factor α; rhIFN-γ, recombinant human IFN-γ; TTBS, 20 mM Tris, 0.05% Tween, and 0.5 M NaCl (pH 7.0); Biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propioamide-biotin; PAR, 4-(2-pyridyalzo) resorcinol; PTIO, 2-phenyl-4,4,5,5-tetramethylimidozoline-1-oxyl-3-oxide.

MATERIALS AND METHODS

Cell Culture. KMBC, a cell line derived from a human cholangiocarcinoma, was used for these studies (24). The cells were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum and maintained at 37°C with 5% CO_2 and 95% humidity.

Transfection of KMBC Cells with hOGG1-1a. The corresponding cDNA for human Ogg1 protein was engineered into mammalian expression plasmid pcDEBΔ-Ogg1a (25). The plasmid was constructed by inserting the corresponding cDNA fragment, to which a sequence for the HA epitope was introduced (25). Transfection of the KMBC cells was carried out using a 4:1 (w/w) ratio of LipofectAMINE Plus (Life Technologies, Inc.):plasmid DNA. The lipid-DNA complexes were overlaid on 70% confluent KMBC cells and incubated at 37°C with 5% CO_2 and 95% humidity in serum-free media for 24 h. After transfection, the cells were incubated for an additional 24 h with complete growth media. The transfection efficiency was determined by transfecting cells with pEGFP-CI (Clontech, Palo Alto, CA) and assessing the percentage of GFP-expressing cells by fluorescence microscopy; the transfection efficiency was approximately 60%. The transfected cells were incubated in the presence of a mixture of inflammatory cytokines (0.5 ng/ml rhIL-1β, 500 units/ml rhTNF-α, and 100 units/ml rhIFN-γ) known to induce iNOS expression in KMBC cells (11), in the presence or absence of 10 mM DTT, a NO scavenger, or 50 μM 1400W [*N*-(3-(aminomethyl)-benzyl)acetamide], a highly selective iNOS inhibitor. The transfected cells were also treated with 300 μM SNAP, a pharmacological NO donor, as a control for the complex signaling events occurring during incubation of the cells with the cytokine mixture. WCEs were prepared as described previously (26, 27).

hOgg1-1a Immunofluorescence in Single Cells. KMBC cells were transfected with HA-hOGG1-1a as described previously. The cells were washed with PBS and fixed in 0.1 M PIPES (pH 6.5), 1 mM EGTA, 3 mM $MgSO_4$, 0.01% gluteraldehyde, and 2% paraformaldehyde at 37°C. The cells were permeabilized in 0.2% Triton X-100 and incubated with a 1:100 dilution of rabbit anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody to bind specifically to the HA-tagged protein and subsequently incubated with a goat antirabbit Texas Red-tagged secondary antibody (1:400 dilution). The cells were mounted and visualized on a fluorescence microscope.

Western Blot Analysis. Cells were harvested by trypsinization and lysed in ice-cold lysis buffer containing 100 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT protease inhibitors (5 mg/ml leupeptin, pepstatin, and chymostatin and 87 mg/ml phenylmethylsulfonyl fluoride), and 1% Triton X-100 for 20 min. Whole cell lysates were boiled in Laemmli buffer [200 mM Tris-HCl (pH 8.0), 8% SDS, 1% bromphenol blue and 400 mM DTT]. Protein samples (40 μg/lane) were loaded on a 7.5% SDS-polyacrylamide gel and separated electrophoretically. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) overnight at 90 mA in a Hoefer Scientific TransBlot cell (Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked with 5% nonfat dried milk in TTBS for 1 h. The primary antibody for HA (Santa Cruz Biotechnology) was applied at a 1:5000 dilution for 2 h. The membrane was washed three times in TTBS for 10 min each before applying the secondary goat antirabbit antibody (Transduction Laboratories, Kensington, KY) at a 1:5000 dilution for 1 h. The blot was washed in TTBS four times for 10 min each. It was then incubated in commercial enhanced chemiluminescence reagent (Amersham, Buckinghamshire, United Kingdom) and exposed to Kodak X-OMAT AR photographic film (Eastman Kodak Company, Rochester, NY).

IP of hOgg1-1a Protein. Cells were harvested by trypsinization and lysed in ice-cold lysis buffer containing 100 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT protease inhibitors (5 mg/ml leupeptin, pepstatin, and chymostatin and 87 mg/ml phenylmethylsulfonyl fluoride), and 1% Triton X-100 for 20 min on ice. After the protein concentration was measured, the HA antibody was added in a ratio of 2:1 by weight, respectively, and cells were incubated with a gentle rolling action overnight at 4°C. The lysate was centrifuged at 13,000 $\times$ *g* for 10 min, and the supernatant was transferred to a dolphin-nosed IP tube (PEG Scientific, Frederick, MD). Sepharose A (0.1 part by volume) and Sepharose G (0.05 part by volume) were added to the supernatant, which was then incubated at 4°C with gentle rolling action for 3 h. The beads and protein mixture were washed with radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS], agitated with rolling action at 4°C, and centrifuged at 600 $\times$ *g*. This step was repeated five times. The HA-tagged protein was recovered by elution with Laemmli buffer and incubation at 85°C for 5 min, followed by centrifugation at 13,000 $\times$ *g* for 1 min at room temperature. The supernatant was then loaded on a 12% SDS-polyacrylamide gel and separated electrophoretically. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) overnight at 90 mA in a Hoefer Scientific TransBlot cell (Hoefer Scientific Instruments). The membrane was first exposed to 0.1% FAST Green FCF (Sigma Chemical Co., St. Louis, MO) in 20% methanol and 5% acetic acid to determine the presence of the single band corresponding to the immunoprecipitated protein. The membrane was destained with 1$\times$ TTBS and blocked with 5% nonfat dried milk in TTBS for 1 h. The membrane was probed with primary antibody for HA (Santa Cruz Biotechnology) as described above.

Treatment of hOgg1-1a Immunoprecipitated Protein with NO and Peroxynitrite. Gaseous NO and peroxynitrite were applied to the protein solution using ultrafiltration cups (Ultrafree-MC; Millipore, Bedford, MA). The protein was placed in the filter cup, and the pharmacological chemical solution producing NO was placed in the filtrate collection tube. SNAP and SNOC were used as NO donors, and peroxynitrite was generated using SIN-1 (Cayman Chemicals, Ann Arbor, MI). To maintain an optimum distance of 2 mm (head space gas volume) between target protein on the cellulose membrane and gaseous producing chemicals in the filtrate, 400 μl of 300 μM SNAP, 100 mM SNOC, or 20 μM SIN-1 solution was used. The cup was closed and incubated for 2 h at 4°C. The NO scavenger, 100 μM C-PTIO, the peroxynitrite scavenger, 40 μM MnTBAP, and the iNOS inhibitor, 50 μM 1400W, were added to the enzyme buffer.

Specific DNA Repair Assay. hOgg1-1a activity was assayed by determining the ability of WCEs and immunoprecipitated hOgg1-1a protein to recognize and excise 8-oxodG. A 24-base DNA oligonucleotide, 5′-GAACTAGTG^{8oxo}GATCCCCCGGGCTGC-3′ (Trevigen, Gaithersburg, MD), containing an 8-oxodG oxidative lesion at position 10 from the 5′ end and its complement were used as substrates. The calculated ratio of enzyme:substrate was ~2.5:1. A total of 50 pmol of the 24-bp oligonucleotide substrate with the 8-oxodG lesion was radiolabeled with 4 μM [α-^{32}P]ATP (6000 mCi/mol; New England Nuclear Life Sciences Products, Boston, MA) using polynucleotide kinase and incubated for 45 min at 37°C. The specific repair activity was assayed by incubating 0.5 pmol of radiolabeled oligonucleotide with 1.5 pmol of its complement in 10 mM Tris (pH 7.5), 1 mM EDTA, and 50 mM NaCl at 37°C for 2 h. Positive control experiments with bacterial 8-oxodG repair enzyme, formamidopyrimidine glycosylase, were run simultaneously. The reaction was loaded onto a 15% acrylamide/7 M urea/1$\times$ Tris-borate EDTA gel. The gel was exposed to a storage screen (PhosphorImager; Molecular Dynamics) for 4 min. Autoradiograms were analyzed using ImageQuant software (Molecular Dynamics).

Assay for Protein *S*-Nitrosylation. *S*-Nitrosylation of hOgg1 was assessed using both an immunochemical assay and a biochemical assay. An immunochemical assay (28) was used to detect the formation of *S*-nitrosylated hOgg1-1a protein. HA-tagged hOgg1-1a protein was immunoprecipitated from control cells and cells treated with SNAP, cytokine mixture, and cytokine mixture plus 1400W. The purified protein was first treated with 20 mM methyl methanethiosulfonate (Pierce, Rockford, IL) for 1 min at room temperature. Next, 25% SDS was added to block free thiols, and the mixture was incubated at 50°C for 20 min. The methyl methanethiosulfonate was then removed by desalting three times with the microBioSpin6 (Clontech) column. The nitrosothiol bonds in the hOgg1 protein were selectively reduced with 1 mM sodium ascorbate to thiols. The thiols were reacted at 25°C for 1 h with 4 mM Biotin-HPDP (Pierce), a sulfydryl-specific biotinylating reagent (29). Because the cysteine biotinylation in this assay is reversible, SDS-PAGE sample buffer was prepared without reducing agent and loaded for electrophoresis on SDS-PAGE gel at room temperature, and the above-mentioned steps were carried out with minimum exposure to light. The biotinylated cysteines were immunoblotted with 3 μg/ml anti-biotin (Pierce) following the Western blot procedure described above.

The Saville method (30) was also used to biochemically detect the formation of *S*-nitrosothiol adducts on cysteine residues in the hOgg1-1a protein. The Saville method works on the principle that protein *S*-nitrosylation adducts replaced with mercuric ions will react with sulfanilamide under acidic conditions to produce a diazonium salt. The formation of this salt can be monitored

spectrophotometrically after its reaction with the aromatic amine (*N*-(1-naphthyl)-ethylenediamine) using an absorbance maxima of 540 nm (ϵ = 50,000 M^{-1} cm^{-1}). Purified hOgg1-1a protein was treated with NO generated from 100 mM SNOC (30 min) and 300 μM SNAP (2 h) and peroxynitrite generated from 20 μM SIN-1 (1 h), using ultrafiltration cups as described above. As demonstrated previously (11, 12, 31), approximately 60–80 μM nitrite/nitrate is generated by this treatment. This method prevents contamination of the target protein solution with decomposition byproducts of SNAP, SNOC, and SIN-1. To 1 mg/ml purified hOgg1-1a protein in 100 mM Tris-Cl (pH 6.8) buffer, an equivalent volume of 1% sulfanilamide dissolved in 0.4 M HCl or an equivalent volume of 0.2% $HgCl_2$ in 1% sulfanilamide dissolved in 0.4 M HCl was added. The protein solution was incubated at room temperature for 10 min and mixed with an equal volume of a 0.02% solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 0.4 M HCl (Greiss reagent). The sample absorbance was then read spectrophotometrically between 400 and 700 nm. The *S*-nitrosylation of the protein was quantified as the difference in absorbance between solution with $HgCl_2$ subtracted from one without $HgCl_2$. The experimental results were standardized with glutathione (γ − Glu-Cys-Gly) as described previously (32).

Quantification of Zinc Release from Purified hOgg1-1a. Approximately 500 μg of hOgg1-1a protein was immunoprecipitated from hOgg1-1a-transfected KMBC cells in 100 mM Tris-Cl (pH 7.0). Gaseous NO was applied to the protein solution through ultrafiltration cups with a M_r 10,000 nominal molecular weight off (Ultrafree-MC; Millipore) as described above. SNAP, freshly prepared SNOC, and SIN-1 were used. SNOC (100 mM) was prepared fresh from an equimolar solution of 100 mM sodium nitrite ($NaNO_2$) and 100 mM cysteine hydrochloride as described previously (31). To maintain optimum distance of 2 mm (head space gas volume) between target protein on the cellulose membrane and NO-producing chemicals in the filtrate, 400 μl of 300 μM SNAP or 100 mM SNOC or 20 μM SIN-1 solution was used. The cup was closed and incubated for 30 min (SNOC), 2 h (SNAP), and 1 h (SIN-1), respectively, at 4°C. After incubation, the protein solution was filtered by centrifugation (30 min at 5000 × *g*) to remove protein. The zinc concentrations in the filtrate were determined by monitoring the complexation of Zn^{2+} by PAR at $A_{500\ nm}$ using $ZnSO_4$ as standards as described previously (31).

RESULTS

Can hOgg1-1a Protein Be Expressed in and Immunoprecipitated from KMBC Cells? A HA epitope-tagged hOGG1-1a cDNA expression vector was transfected into KMBC cells, and the protein expression was assayed by both single cell immunofluorescence and immunoblot analysis 48 h later. As assessed by single cell immunofluorescence, transfected KMBC cells expressed the hOgg1-1a protein, which was detected exclusively in the nucleus (Fig. 1*A*); thus, the HA-tagged hOgg1-1a protein was expressed and appropriately localized in the nucleus of transfected cells. We had previously established that these cells express iNOS and generate NO after stimulation with a cytokine mixture (11, 12). To ensure that cytokines did not influence the expression of hOgg1-1a, we transfected cells in the presence of these compounds. Cytokine mixture and 1400W, a specific iNOS inhibitor, had no effect on hOgg1-1a protein expression after transfection as assayed by immunoblot analysis (Fig. 1*B*). Purified protein was obtained by IP using HA antibody. The isolated protein was analyzed by Western blot to determine the purity of the IP procedures. Only a single M_r 36,000 protein band corresponding to HA-tagged hOgg1-1a was identified on the blot with preliminary FAST Green FCF staining (data not shown) and confirmed with HA immunoblotting (Fig. 1*C*). Thus, hOgg1-1a can be efficiently expressed and immunoprecipitated from this cholangiocyte-derived cell line.

Does NO Inhibit the BER Activity of hOgg1-1a? A 24-bp oligonucleotide with a single 8-oxodG lesion was used as a substrate for hOgg1 activity. BER activity of WCEs after transfection was found to be equally efficient as that of untransfected cells, demonstrating endogenous expression of hOgg1 in KMBC cells. However, when cells were treated with cytokine mixture (0.5 ng/ml rhIL-1β, 500 units/ml rhTNF-α, and 100 units/ml rhIFN-γ) to induce iNOS or treated with SNAP, a pharmacological NO donor, BER activity was inhibited (Fig. 2*A*). Coincubation with DTT, a scavenger for NO, or 1400W, an iNOS inhibitor, prevented loss of BER activity. (Fig. 2*A*). To more directly examine the effects of NO on hOgg1 activity, HA-tagged hOgg1-1a protein was immunoprecipitated from cells transfected with its expression vector. Immunoprecipitated hOgg1-1a protein isolated from cells incubated with cytokine mixture was unable to excise 8-oxodG lesions (Fig. 2*B*). In contrast, immunoprecipitated hOgg1-1a from cells incubated with cytokine mixture in the presence of 1400W manifested normal BER activity (Fig. 2*B*). These data indicate that NO generated from iNOS inhibited hOgg1-1a activity.

Is the BER Activity of hOgg1-1a Inhibited by NO and/or Peroxynitrite? Next, we examined the effects of NO and peroxynitrite on hOgg1-1a activity. The BER activity of immunoprecipitated hOgg1-1a was assayed after treatment with SNAP, a NO donor, and SIN-1, a chemical that releases NO and superoxide (which combine to form peroxynitrite). When the enzyme was exposed to SNAP or SIN-1, its activity was inhibited. However, when C-PTIO, a NO scavenger, was added to the enzyme buffer, activity was preserved (Fig. 3, *A* and *B*). MnTBAP, a superoxide mimetic, was also effective in preventing inhibition of enzyme activity during treatment with SIN-1 (Fig. 3*B*). The results suggest that either NO or peroxynitrite is capable of inhibiting the BER activity of hOgg1.

Does NO Directly Nitrosylate hOgg1-1a? An immunoblot assay was initially used to detect sites of NO-thiols on the hOgg1 protein. This assay labels these sites with Biotin-HPDP. Immunoblot analysis for Biotin-HPDP on hOgg1-1a identified *S*-nitrosothiol sites on the protein from both cytokine- and SNAP-treated cells. The nitrosylation of hOgg1 was NO dependent in the cytokine-treated cells because 1400W prevented labeling.

Immunoprecipitated hOgg1-1a was treated with SNOC, SNAP, and SIN-1, and the number of *S*-nitrosothiol adducts was determined using the Saville reaction. Approximately 0.24, 0.30, and 0.17 μM *S*-nitrosothiol adducts form per milligram of purified hOgg1-1a protein after treatment with SNOC, SNAP, and SIN-1, respectively (Fig. 4*B*). Control experiments with untreated protein demonstrated that essentially no *S*-nitrosothiol adducts were formed. Thus, NO is capable of directly nitrosylating hOgg1.

Is NO-mediated *S*-Nitrosylation of hOgg1-1a Accompanied by Loss of Zn^{2+}? Free zinc released from immunoprecipitated hOgg1-1a protein was assayed using the metallochromic indicator PAR. This compound, when complexed to zinc, produces a visible absorption maximum at 500 nm (ϵ = 66,000 M^{-1} cm^{-1}). Using this experimental approach, we estimated the release of 0.58, 0.61, and 0.38 μg of Zn^{2+} from 500 μg of HA-tagged αhOgg1 treated with SNOC, SNAP, and SIN-1, respectively (Fig. 5). Minimal release of zinc was observed in control experiments. These data suggest that nitrosylation of hOgg1 results in expulsion of zinc from its zinc finger motif configuration.

DISCUSSION

The results of the current study relate to NO-mediated inhibition of BER by hOgg1. The results demonstrate that (*a*) NO generated via cytokine induction of iNOS results in loss of hOgg1-1a BER activity, (*b*) hOgg1-1a enzyme activity can be directly inhibited by NO and peroxynitrite donors in a cell-free system, and (*c*) NO donors result in the formation of hOgg1-1a *S*-nitrosothiol adducts and loss of protein-bound zinc ions. The current studies suggest NO directly nitrosylates hOgg1, a reaction that causes loss of bound zinc and irreversible loss of enzyme activity. In conjunction with our previous studies (11, 12),

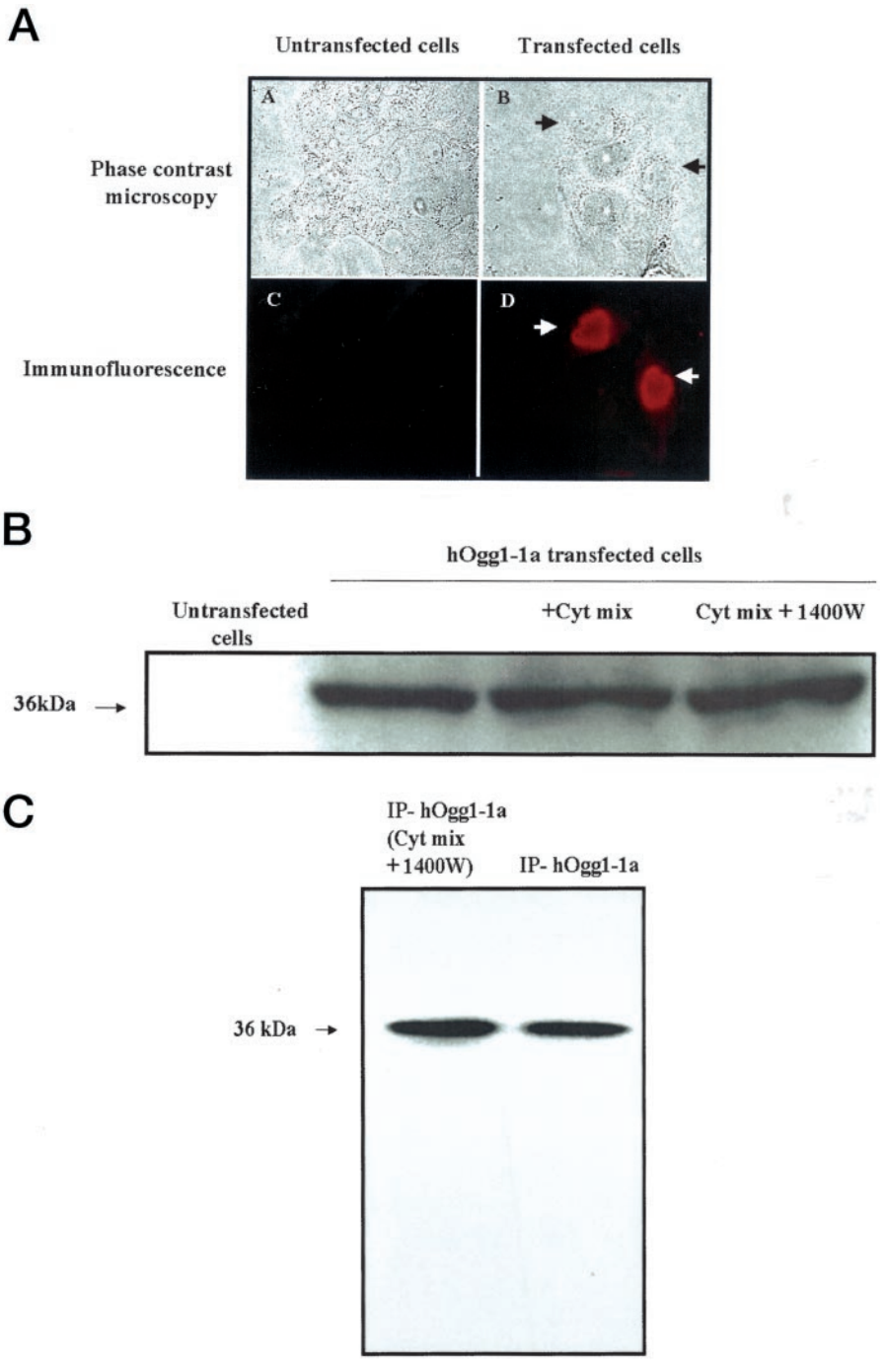

Fig. 1. hOgg1-1a protein is expressed in KMBC cells after transfection and isolated by IP. KMBC cells were transfected with an expression vector for HA epitope-tagged hOGG1-1a. *A*, cells were identified by phase-contrast microscopy (*panels A* and *B*). Transfection location (nuclear) and efficiency were visualized by immunocytochemistry with primary antibody against HA followed by secondary fluorescent Texas Red tag (*panel D*). Untransfected cells did not express nuclear HA-tagged hOgg1-1a protein (*panel C*). *B*, 24 h after transfection, the cells were harvested, and hOgg1-1a protein expression assayed by HA immunoblot analysis. The protein expression was not affected by incubation with cytokine mixture (0.5 ng/ml rhIL-1β, 500 units/ml rhTNF-α, and 100 units/ml rhIFN-γ) for 24 h with or without 50 μM 1400W, a specific iNOS inhibitor. *C*, HA antibodies were used to isolate the hOgg1-1a protein by IP as described in "Materials and Methods." The isolated hOgg1-1a protein was assayed by Western blot, and a corresponding single M_r 36,000 protein band was identified. Incubation with cytokine mixture or 1400W did not alter the levels of protein isolated by this procedure.

these results provide strong biochemical data to further implicate NO as an inhibitor of cellular BER activity.

We have previously shown that proinflammatory cytokines inhibit BER activity in cholangiocytes and cholangiocarcinoma cells by a NO-dependent process. The current study significantly extends these observations by identifying hOgg1 as a molecular target for NO inhibition of BER activity. Furthermore, the data provide mechanistic insight into NO-mediated inhibition of hOgg1 activity. Studies by others have shown that NO can reversibly or irreversibly inhibit their catalytic activity (21, 33, 34). NO inhibition of glycosylases has been attributed to *S*-nitrosothiol and 3-nitrotyrosine adduct formation (22, 35). The present data demonstrate the formation of *S*-nitrosothiol adducts in hOgg1-1a protein after treatment with NO donors. In contrast, 3-nitrotyrosine residues could not be detected by immunoblot analysis in the experimental paradigms used (data not shown). The formation of these *S*-nitrosothiol adducts was also associated with the loss of zinc ions from the protein. The release of zinc ions measured in response to *S*-nitrosothiol formation strongly suggests that loss of the active site cysteine 4-type zinc finger motif causes irreversible loss of hOgg1 tertiary structural integrity and hence loss of enzyme function.

The present findings suggest that either NO or peroxynitrite may inhibit hOgg1. For example, SNAP, a pharmacological NO donor, and SIN-1, a peroxynitrite donor, both inhibited hOgg1 activity. These results were bolstered by showing that C-PTIO, a NO scavenger, and MnTBAP, a peroxynitrite scavenger, protected the enzyme from loss of activity by SNAP and SIN-1, respectively. These results indicate that NO generation during inflammation may be sufficient to inhibit

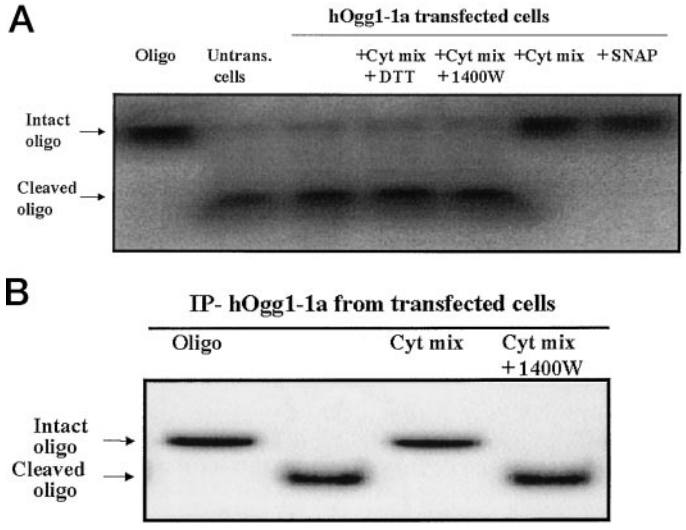

Fig. 2. NO generated from increased iNOS expression after cytokine stimulation inhibits BER activity of hOgg1-1a. 8-oxodG-specific BER activity is assayed by recognition and excision of the 8-oxodG lesion from the 24-bp oligonucleotide giving the 15-bp cleaved product. *A,* WCEs from untransfected and hOGG1-1a-transfected cells exhibited efficient BER activity. Incubation with cytokine mixture or 300 μM SNAP for 24 h after transfection resulted in an inability to excise the 8-oxodG lesion. Coincubation with 50 μM 1400W or 10 mM DTT resulted in efficient excision activity. *B,* hOgg1-1a protein was isolated from cells that were incubated with cytokine mixture $\pm$ 50 μM 1400W by HA IP. BER activity of purified hOgg1-1a protein was inhibited in cells incubated with cytokine mixture and normal in cytokine $\pm$ 1400W-treated cells.

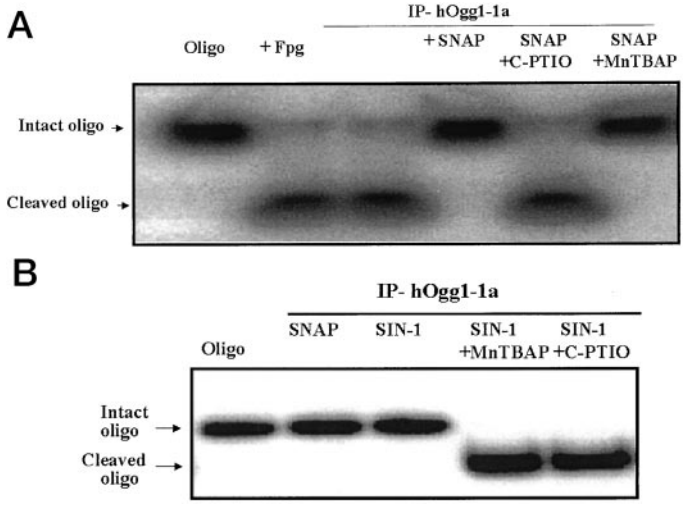

Fig. 3. NO and peroxynitrite generated from chemical donors inhibit BER activity of hOgg1-1a. 8-oxodG-specific BER activity is assayed by recognition and excision of the 8-oxodG lesion from the 24-bp oligonucleotide giving the 15-bp cleaved product. Formamidopyrimidine glycosylase (Fpg), a bacterial 8-oxodG DNA-glycosylase, was used as control. *A,* hOgg1-1a isolated by HA IP from KMBC cell lysates and exposed to NO from 300 μM SNAP did not excise the 8-oxodG lesion. This inhibition was ineffective in the presence of C-PTIO but not MnTBAP. *B,* hOgg1-1a protein exposed to peroxynitrite generated from 20 μM SIN-1 had inhibited BER activity that was reversed in the presence of MnTBAP and C-PTIO.

repair activity, but peroxynitrite generated from NO and superoxide in inflamed tissues may also contribute to inhibition of this enzyme. Scavengers for both reactive nitrogen species are therefore expected to help preserve base excision DNA repair activity in chronic inflammatory diseases.

We acknowledge that our results are based largely on *in vitro* biochemical assays. Optimally, a specific assay for hOgg1 activity in living cells would be ideal to confirm these observations. However, a robust assay specific for specific hOgg1 activity in living cells has not been developed and is difficult to conceive, given current technological limitations. Therefore, extrapolation of our data to tissues *in vivo* must take these considerations into account.

The *hOGG1* gene maps to chromosome 3p26.2. This region shows loss of heterozygosity in a variety of human cancers accompanied by frequent GC→TA transversions and may therefore function as a tumor repressor (15, 18, 36). Mutations in the *hOGG1* gene have also been identified in gastric cancer (37) and in lung and kidney tumors (38), further suggesting that loss of hOgg1 function contributes to the carcinogenesis process. Our previous studies, along with the current findings, suggest that in addition to mutations in the *hOGG1* gene, posttranslational modification of hOgg1 may also contribute to the initiation, promotion, and progression of human cancers. The latter mechanisms may be especially important in chronic inflammatory

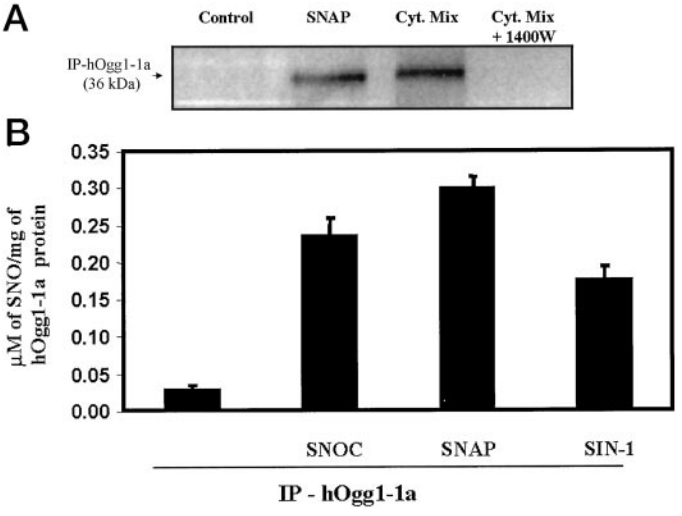

Fig. 4. hOgg1 can be directly nitrosylated by NO and/or ONOO. *A,* cells were transfected with HA-hOgg1 and then treated with SNAP (300 μM), cytokine mixture, and cytokine mixture plus 1400W (50 μM) for 24 h. Cell lysates were then obtained, and the HA-tagged hOGG1 protein was immunoprecipitated. Sites of nitrothiol adducts were labeled with Biotin-HPDP as described in "Materials and Methods." The protein was separated by PAGE. The Biotin-HPDP-labeled sites were identified by an immunoblot assay. Purified hOgg1-1a protein isolated from control cells and cells treated with 300 μM SNAP, cytokine mixture, and cytokine mixture + 50 μM 1400W was subjected to *S*-nitrosylation assay, resolved by SDS-PAGE, and immunoblotted with an antibiotin antibody. Only *S*-nitrosylated hOgg1-1a protein was capable of producing a signal in this assay. *B, S*-nitrosothiol adducts in hOgg1-1a were detected following a modified Saville method as described in "Materials and Methods." Purified hOgg1-1a protein did not contain any measurable amounts of *S*-nitrosothiols. Exposure of the protein to NO generated from 300 μM SNAP for 2 h and 100 mM SNOC for 30 min resulted in a significant amount of *S*-nitrosothiol adducts. Formation of peroxynitrite with 20 μM SIN-1 (1 h) also generated *S*-nitrosothiol adducts to a lesser extent.

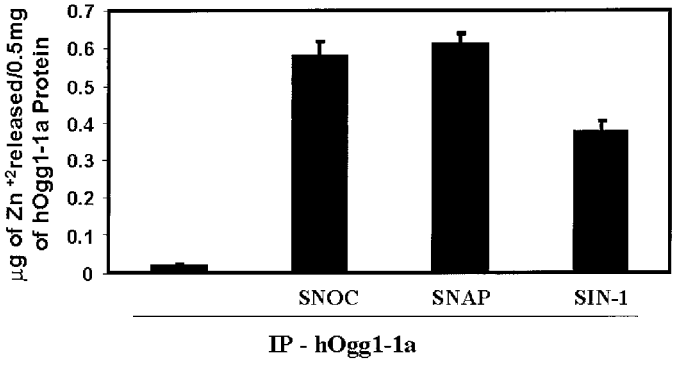

Fig. 5. *S*-Nitrosothiol adducts in hOgg1-1a are accompanied by loss of zinc. A colorimetric procedure was used to detect the PAR-Zn complex formed after the release of zinc from hOgg1-1a. Exposure of the protein to NO generated from 300 μM SNAP for 2 h and 100 mM SNOC for 30 min resulted in the release of a significant amount of free zinc. Formation of peroxynitrite with 20 μM SIN-1 (1 h) also caused the release of zinc.

diseases in which iNOS is induced and relatively high concentrations of NO are generated. This concept suggests that iNOS inhibition would have merit as a chemopreventive strategy to decrease cancer development in chronic inflammatory diseases of the biliary tract and perhaps in other organs as well. Chemopreventive approaches may include inhibition of iNOS expression with minocycline (39), inhibition of iNOS activity with selective nontoxic inhibitors (40, 41), or administration of NO scavengers (42). Animal models of carcinogenesis and cancer progression in wild-type and iNOS knockout animals coupled with studies of BER activity of hOgg1 will be useful to further test these concepts.

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