Inactivation and Degradation of O6-Alkylguanine-DNA Alkyltransferase after Reaction with Nitric Oxide

Liping Liu, Meng Xu-Welliver, Sreenivas Kanugula, and Anthony E. Pegg

Department of Cellular and Molecular Physiology, The Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

ABSTRACT

O6-Alkylguanine-DNA alkyltransferase (AGT) plays a critical role in protection from the carcinogenic effects of simple alkylating agents by repairing O6-alkylguanine adducts via a direct transfer reaction. Nitric oxide (NO) or species derived from it are known to be able to initiate neoplastic growth and cannot only damage DNA, either directly or via the formation of intermediates such as nitrosamines, but can also inhibit some DNA repair processes. We have studied the inactivation of AGT by NO in detail in vitro and in vivo using wild-type human AGT (hAGT) and mutants at key residues. Our results show that hAGT is readily but reversibly inactivated by the formation of S-nitrosocysteine at Cys-145, which is the alkyl acceptor site. The facile reaction of this cysteine residue with NO is attributable to its interaction with other residues in hAGT including His-146 and Glu-172 that activate the sulphydryl group of Cys-145 to allow its nucleophilic attack on DNA adducts. Although the S-nitrosocysteine adduct in hAGT is readily reversible by reaction with other cellular thiols, the formation of S-nitrosocysteine at Cys-145 was found to lead to the rapid degradation of the hAGT protein in vitro. This degradation is brought about by the ubiquitin/proteasomal system. The formation of an S-nitrosocysteine at Cys-145 in hAGT in response to NO led to a large increase in the ubiquitination of the protein. This NO-mediated increase did not occur with the C145S or C145A mutants. A conformational change in hAGT, which involves opening of an asparagine hinge, normally occurs after alkylation of the protein in its role in DNA repair and causes degradation of the alkylated hAGT. Our results indicate that a similar effect occurs after reaction of the protein with NO. Thus, exposure to NO causes an irreversible loss of DNA repair capacity for alkylation adducts. This may contribute toward the potential development of tumors in cells upon chronic exposure to NO because of inflammation or infection. This may be of particular importance because such exposure may also lead to the formation of N-nitroso compounds that can act as alkylating agents.

INTRODUCTION

The reaction of alkylating agents with DNA forms many different adducts that probably all contribute to their effects (1–3). Adducts formed at the O6 position of guanine are of major importance in both the initiation of mutations and in the cytotoxic actions of these agents. The predominant mechanism for repair of this adduct is via the action of AGT, and many studies have shown that this AGT protein therefore forms an important protective mechanism against the mutagenic, carcinogenic, and cytotoxic effects of simple alkylating agents (4–7).

AGT repairs O6-alkylguanine in double-stranded DNA by transferring the alkyl group from the DNA to an internal cysteine residue in the AGT protein. A probable molecular mechanism of DNA repair by AGT is suggested by studies of its crystal structure and models of substrates bound to the protein (8). The DNA is bound via a winged helix-turn-helix DNA binding domain, and the target O6-alkyloxoguanosine is flipped out of the DNA helix and is placed in a binding pocket that contains the cysteine acceptor site. This cysteine, which is residue Cys-145 in hAGT, is part of an extensive hydrogen-bonding network involving a well-ordered water molecule and residues Tyr-158, His-146, and Glu-172. Proton abstraction to facilitate nucleophilic attack by Cys-145 is likely to occur through this hydrogen-bond network. The activated cysteine residue then attacks the O6-alkyl group forming S-alkylcysteine and restoring the DNA structure. The S-alkylcysteine formed at the active site of AGT is not converted back to cysteine, so the protein can act only once. The alkylated form of the AGT protein undergoes a conformational change, which leads to its rapid degradation, probably by facilitating its ubiquitination (9–12).

NO has many roles in mammalian physiology including those as a second messenger regulating blood flow, thrombosis, and neural activity. It is also important in host defenses against pathogens. However, it is also known that excessive or prolonged exposure to NO may play a causative role in a variety of pathophysiological processes including carcinogenesis (13–15). Previous studies have indicated that exposure to an agent generating NO led to a loss of AGT activity in rat H4 cells and in purified RAGT (16). Although it was suggested that an N-nitrosothiol adduct was responsible for this inactivation, this mechanism was not established. Furthermore, because S-nitrosothiol adducts would be expected to be readily reversible by reaction with other cellular thiols, the extent to which this inactivation would be prolonged was unclear. Therefore, we have examined in detail the interaction of hAGT with NO and the fate of the inactivated protein. Our results show that inactivation occurs via reaction with the Cys-145 acceptor residue. The facile formation of S-nitrosocysteine at this site causes a configurational change leading to ubiquitination similar to that produced by an alkyl group in the AGT-mediated repair reaction. The nitrosylated form of hAGT is therefore rapidly degraded, and exposure to NO causes an irreversible loss of DNA repair capacity. This loss may contribute toward the potential development of tumors in cells having prolonged exposure to NO. An abstract describing this work has been published (17).

MATERIALS AND METHODS

Materials. SNAP, GSNO, α-cyano-4-hydroxycinnamic acid, and most other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All restriction enzymes, alkaline phosphatase, and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). The pQE30 vector and the DNA isolation kits were from Qiagen (Chatsworth, CA). Immobilized trypsin was from Roche (Indianapolis, IN). Phototope-HPRT Detection Kit for Western blotting analysis was from Cell Signaling Technology (Beverly, MA). [35S]Methionine was obtained from DuPont (Boston, MA). Ubiquitin aldehyde was obtained from Boston Biochemicals (Cambridge, MA). Rabbit reticulocyte lysate prepared from phenyl-hydrazine-treated New Zealand White rabbits was obtained from Cocalico Chemicals (Cambridge, MA). Rabbit reticulocyte lysate prepared from phenyl-hydrazine-treated New Zealand White rabbits was obtained from Cocalico Biologicals (Reamstown, PA). MG132 was purchased from Calbiochem (La Jolla, CA) and dissolved in ethanol at a 10 mM stock solution. POROS 20MC metal chelate affinity medium and POROS HS cation exchange medium were purchased from PerSeptive Biosystem (Framingham, MA).
Plasmids. The plasmids used for protein expression, which have been described previously, were: pGEM-SSAT (18); pCMZEO-AGT and pCMZEO-C145S (12); pCMV-AGT and pCMV-C145A (19); pQE30-AGT (20); pIN-Y114E (21); and pQ-E-Y158H (22). A plasmid for expression of the Y114E-Y158H double mutant was made by digesting pIN-Y114E mutant with EcoRI and BamHI and purifying the 600-bp fragment. After further digestion with EcoNI and DraIII, the fragment was ligated into the pQE-Y158H vector digested with the same enzymes to form pQE-Y114E/Y158H. The entire sequence of this coding region was confirmed by DNA sequencing.

Expression and Purification of COOH-Terminal Histidine-tagged hAGT. A plasmid vector for the preparation of a recombinant COOH-terminal His-tagged hAGT (AGT-His6) in which the six terminal amino acids were replaced with histidine residues was constructed from pQE30-AGT (20) by PCR. Oligonucleotides 5'-CACACAAGATTCATTAAAGAGGAAATTAACATGGCAAGATGTGTTG-3' with an EcoRI site preceding the start codon and 5'-ACCCGGGTATCCACTCAGTGATGTTGATGGTGAGCCGGAGTGAC-3' with a KpnI site following the stop codon were used as the sense strand primer and antisense strand primer, respectively (restriction sites are underlined). The purified PCR product was digested with EcoRI and KpnI. The resulting fragment containing the modified AGT sequence was ligated into a pQE30 vector digested with the same enzymes and transformed into XL1-blue cells (Stratagene). The entire sequence encoding the hAGT protein was verified by DNA sequencing at the Macromolecular Core Facility, Hershey Medical Center, and it corresponded to the sequence for the normal protein. The purity of the expressed AGT was assessed by Coomassie Blue staining.

The recombinant protein was expressed in XL1-Blue cells and was purified via BioCAD Purification System (PerSeptive Biosystems) to high homogeneity (>99%) as estimated by PAGE. XL-1 Blue cells expressing the AGT-His6 were lysed with B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). Cell membrane debris was removed by centrifugation twice at 12,000 rpm for 15 min at 4°C. Supernatants from the second spin were loaded onto a CoCl2-charged (pH 4.8) POROS 20MC column connected to the BioCAD Purification System. The column was washed extensively with 20 mM HEPES (pH 8.0) and 500 mM NaCl buffer. The AGT-His6 protein was eluted with a buffer containing 20 mM HEPES (pH 7.6), 500 mM NaCl, and 100 mM imidazole. The elute was diluted 10-fold with 20 mM HEPES (pH 7.2), 0.1 mM EDTA, and 5 mM DTT and loaded onto a POROS HS cation exchange column. Bound proteins were eluted using 20 mM HEPES buffer (pH 7.2) with 50–1000 mM NaCl gradient over 30 min, and the hAGT protein was eluted around 0.45–0.5 M NaCl. The salt contents in the AGT-His6-containing fractions were reduced to <50 mM by dilution with AGT buffer [50 mM Tris (pH 7.6), 0.1 mM EDTA, and 5 mM DTT], and the protein was concentrated by CentriTec (Millipore, Bedford, MA) centrifugation. The AGT-His6 was also purified by TALON resin chromatography (Clontech, Palo Alto, CA) as described previously (20). However, as shown in Fig. 1, the protein prepared via the POROS 20MC metal chelate affinity column followed by a cation exchange chromatography lacks the impurity proteins of M, 15,000 and M, 45,000 obtained using the TALON resin chromatography.

AGT Activity Assay. AGT activity was determined by measuring the transfer of radioactive from O3-[3H]methyl guanine in cDNA to AGT protein (23). Briefly, an aliquot of hAGT (0.02 μg) was incubated with 1H-methylated DNA substrate in AGT buffer for 30 min at 37°C. The reaction was stopped by the addition of a denaturation buffer containing 8 M urea, 20 mM Tris (pH 7.6), and 60 mM NaCl. Radioabeled AGT protein was recovered by filtration through 0.45 μm nitrocellulose membranes. The amount of 1H bound to AGT was determined by scintillation counting.

Treatment of hAGT with Compounds Releasing NO. hAGT (0.02–0.06 μg) was added to 0.5 ml of buffer [50 mM Tris (pH 7.6) and 1 mM EDTA] containing 0.5 mM SNAP or GSNO. The reaction was allowed to proceed in the dark for 30 min at 50 μg chymotrypsin at 30°C. Residual AGT activity was then assessed. The reversibility of the reaction between hAGT and SNAP was examined by dilution 1:3 (v/v) with buffer and incubation with 10 mM DTT for an additional 30 min before hAGT activity was assessed.

Mass Spectrometry. The AGT-His6 (1 nmol) and its reaction products with SNAP were digested with 20 μg of immobilized trypsin in 200 mM ammonium chloride (pH 8.0) at 50°C for 2 h. The ammonium ion in the reaction mixtures was removed by aspirating and desipering from ZipTipC18 pipette tip (Millipore). hAGT peptides were eluted from tips using 30, 50, 70, and 100% acetonitrile trifluoroacetic acid (0.1%) solutions. α-Cyano-4-hydroxycinnamic acid [8 mg/ml in 50% acetonitrile aqueous trifluoroacetic acid (0.3%) solution] was used as matrix. Mass analysis was performed using a Voyager BioSpectrometry matrix-assisted desorption ionization-time of flight Workstation (PerSeptive Biosystem) with laser attenuation 400 and an accelerating voltage of 19,725 V. The resulting spectra were analyzed using Grams software.

Cell Treatment. CHO cells stably transfected with plasmids expressing the wild-type hAGT or its C145A mutant driven by the CMV promoter were cultured as described previously (19). Cells were plated at 6 × 104 cells/cm2 24 h before treatment at ~80% confluence. They were treated with 0–15 mM SNAP or GSNO for 2 h. Control cells were treated with 0.5% DMSO. AGT activities in 20-μg cell extracts were measured as described previously (19). To study the stability of hAGT after exposure to NO, CHO cells expressing wild-type hAGT or the C145A mutant were treated for 1 h with 10 mM SNAP in the presence or absence of 25 μM MGI132. Cycloheximide (200 μM) was then added to the medium, and cells were harvested at 0, 0.5, 1, 2, and 4 h later.

Western Blotting Analysis. Nitration of tyrosine residues in purified hAGT protein was examined by Western blotting using a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Wild-type hAGT, hAGT mutants, or superoxide dismutase (2 μg) was incubated with 5 mM SNAP or DMSO vehicle control at 37°C for 30 min. Reaction mixtures were diluted 1:3 and were incubated with 10 mM DTG for 30 min. Reaction products were separated by 15% PAGE and transferred to a polyvinylidene difluoride membrane. The detection of nitrosotyrosine was carried out using an anti-nitrotyrosine antibody.

Levels of hAGT and C145A proteins in CHO cells with or without SNAP treatment were examined by Western blotting using a monoclonal antibody to hAGT. Crude cell extracts from hAGT-expressing CHO cells were prepared in the presence or absence of 5 mM NEM (Sigma) as described previously (24). Aliquots (20 μg) of total cell lysate separated on 15% SDS-PAGE were probed with monoclonal methylguanine DNA methyltransferase Ab-1 from NeoMarkers (Fremont, CA) and monoclonal actin antibody from Oncogene (Boston, MA). The density of protein bands was quantitated by NIH Image 1.61 software.

AGT Degradation in Reticulocyte Lysates. Preparations of hAGT, the C145S mutant, or SSAT labeled with 35S-methionine was synthesized from 0.5 μg of plasmid pCMZEO-AGT, pCMZEO-C145S, or pGEM-SSAT in a coupled transcription/translation system (12). The proteins were then incubated with 5 mM GSNO or water alone (control) for 1 h at 37°C. Each reaction mixture was partially purified by ammonium sulfate precipitation and by using a G50 Sephadex quick spin column to remove excess GSNO. Degradation of each partially purified TNT product 35S-labeled protein was studied by incubation with 2–μl aliquots with a crude rabbit reticulocyte lysate in a standard assay volume of 0.1 ml at 37°C containing 40 mM Tris/HCl (pH 7.5), 5 mM MgCl2, 2 mM ATP, 10 mM cycloheximide, and 25 μl of reticulocyte lysate.
experiments, 141 \mu M ubiquitin, 5 \mu M ubiquitin aldehyde, and 100 \mu M MG132 were also present. Aliquots of 45 \mu l of the degradation mix were removed, mixed with SDS, and boiled for 10 min before separation by SDS-PAGE. The rate of \({}^{35}\)S-labeled hAGT degradation was determined by quantifying the radioactivity of the M_2 22,000 band corresponding to hAGT on dried gels using a Molecular Dynamics PhosphorImager SI and ImageQuant application software. The level of conjugation was measured the same way by quantifying the area of the ladder of ubiquitinated protein bands above each main band.

RESULTS

Generation of Recombinant hAGT. Although an NH\(_2\)-terminal histidine-tagged recombinant hAGT was made previously by addition of a 12-amino acid sequence, MRGSHHHHHHGS, to the NH\(_2\) terminus of the protein (20), studies of this protein have shown that the addition of the NH\(_2\)-terminal tag may slightly impair its alkyltransferase activity. As shown in Table 1, the second order rate of repair for the NH\(_2\)-terminal histidine-tagged hAGT was approximately half of that of nontagged hAGT. Because deleting the COOH-terminal residues 177–207 in hAGT did not affect alkyltransferase activity (25, 26), a COOH-terminal histidine-tagged hAGT was made by replacing the COOH-terminal six amino acids (PPAGRN) with histidines. The activity of this AGT-His\(_8\) protein was comparable with the nontagged hAGT (Table 1), and it was used for all \textit{in vitro} experiments described in this report.

Inactivation of hAGT \textit{in Vitro}. Incubation of AGT-His\(_8\) with SNAP or GSNO inactivated the protein in both a time- and dose-dependent manner (Fig. 2, A and B). The ED\(_{50}\) for hAGT inactivation by SNAP and GSNO (Fig. 2B) are 400 and 800 \mu M, respectively. The hAGT activity was reduced by \(~95\%\) after 30-min exposure to 5 mM SNAP. Addition of ctDNA to the reaction retarded hAGT inhibition by SNAP, suggesting that hAGT is less reactive with NO when it binds to DNA (Fig. 2C).

Addition of the thiol-reducing agent DTT reversed up to 70\% of the SNAP-mediated inhibition of hAGT within 30 min (Fig. 2C). Because the S-NO bond can be readily reduced by this reagent, leading to the recovery of alkyltransferase activity, this suggests that the inactivation is brought about by reaction with a sulfhydryl group in hAGT. Although hAGT contains five cysteines, the most likely candidate for this is Cys-145, which serves as the alkyl acceptor. The S-nitrosylation of Cys-145 was confirmed by matrix-assisted desorption ionization time-of-flight analysis of the hAGT after reaction with SNAP. Unreacted hAGT protein and hAGT SNAP reaction product were digested with immobilized trypsin before mass analysis. Complete tryptic digest would give rise to seven hAGT peptides which have masses between 1000 Da to 2000 Da. Six of the seven hAGT peptide fragments, including peptides 108–125, 136–147, 148–165, 166–175, and 176–193 (Fig. 3), were observed from the 50\% acetonitrile ZipTip eluate. One signal with an m/z ratio of 1314.4, which corresponds to residues Gly-136-Arg-147, was observed for the unreacted hAGT was shown to be recognized by an antiserum specific for nitrotyrosine (Fig. 4A). Such reaction could also cause a loss of activity because two of the three tyrosine residues in hAGT (Tyr-114 and Tyr-158) are highly conserved in AGTs and are known to be present in the DNA binding domain and active site pocket (8). However, the hAGT mutants Y114F, Y158H, and the double mutant Y114E/Y158H showed a similar level of reaction with the antiserum after reaction with SNAP (Fig. 4B), suggesting that the most likely site for nitration is at Tyr-68. Tyr-68 is a surface residue, which would increase its chances of reacting with NO, but it is not located near the active site and is not conserved in AGTs; therefore, it is unlikely to play a role in the reaction mechanism.

Inactivation and Degradation of hAGT \textit{in Vivo}. To study the reaction of hAGT with NO \textit{in vivo} and to use mutants of the protein, the hAGT was expressed in CHO cells, which lack endogenous AGT.
Stable clones expressing hAGT from a plasmid with a CMV promoter were used (19). After a 2-h treatment of such cells with SNAP or GSNO, up to 95% of the alkyltransferase activity was lost in a dose-dependent manner (Fig. 5). This result provides evidence that high concentrations of NO can inhibit hAGT in the cellular environment, even in the presence of endogenous reducing reagents.

Previous studies have shown that hAGT has an increased susceptibility to proteolysis after alkylation of Cys-145. This protein is rapidly ubiquitinated and degraded after alkylation, both in intact cells and in cell extracts (9, 10). In contrast, unreacted hAGT is relatively stable. To investigate the stability of hAGT-inactivated by NO, the half-life of the protein in CHO cells stably transfected with pCMV-hAGT was measured. After treatment with 10 mM SNAP for 1 h, cycloheximide was then added to block protein synthesis, and cells were harvested at various times. The hAGT content in cell extracts was determined by Western blotting analysis using a monoclonal antibody to hAGT. There was only a moderate loss of hAGT protein in vehicle-treated cells within 4 h after treatment, but there was a substantial loss of protein in cells treated with SNAP (Fig. 6A). In contrast, there was no observed loss of actin when the same membrane was probed with an anti-actin antibody, although this protein is known to react with NO (27). Levels of hAGT protein in cell extracts normalized with the amounts of actin were expressed as a percentage hAGT remaining and plotted against time after treatment in Fig. 6B. The $t_{1/2}$ of hAGT was reduced to 1.3 h after SNAP treatment. [The decline in hAGT protein in the absence of SNAP was not large enough for a half-life to be estimated accurately in this experiment, but previous studies have shown that wild-type hAGT has a $t_{1/2}$ of >24 h in cells unexposed to alkylating agents (9–12).] The addition of proteosome inhibitor MG132 together with SNAP to the cultures significantly blocked hAGT protein loss (Fig. 6, C and D).

A ladder of protein bands, representing the hAGT-ubiquitin complexes, was observed in Western blotting of cell extracts developed using the anti-AGT antibody (Fig. 7). It was necessary to include...
NEM in the lysis buffer to prevent the break down of ubiquitinated forms of hAGT during cell extract preparation to maximize the retention of these conjugates (10). Reaction with NO significantly increased the levels of ubiquitinated forms of hAGT in CHO cell extracts prepared from cells exposed to MG132 to block their proteasomal degradation (Fig. 7). Treatment of CHO cells with MG132 in the absence of SNAP (Fig. 7, left side) or alkylation damage does not lead to the appearance of ubiquitinated forms of hAGT (12).

Mutation of Cys145 to Ala completely abolished the enhancement of hAGT degradation upon reaction with NO. Exposure to SNAP of CHO cells stably transfected with pCMV-C145A did not affect the degradation of the C145A hAGT protein, which remained as stable as unreacted wild-type hAGT (Fig. 8). This result suggests strongly that it is the formation of S-nitrosocysteine at Cys-145 that leads to the enhanced ubiquitination and degradation of hAGT.

Ubiquitination of hAGT in Vitro. The ubiquitination of many rapidly turning-over proteins including hAGT can be studied in a reticulocyte lysate system using 35S-labeled protein prepared from a coupled transcription/translation reaction (18). The level of hAGT ubiquitin conjugation is maximized in this lysate in the presence of an ATP regeneration system, MG132 to block degradation by the 26S proteasome, and ubiquitin aldehyde to inhibit ubiquitin COOH-terminal hydrolases, particularly isopeptidase T (28, 29). As shown in Fig. 9, exposure to GSNO substantially increased the formation of a ladder of ubiquitinated forms of wild-type hAGT. The percentage of ubiquitin-hAGT conjugates was increased from 12 to 33% of the total protein after such treatment. There was a small level of conjugates present at zero time in this experiment, and it increased with incubation even in the absence of NO. This may be attributable to the ubiquitination of improperly folded hAGT proteins during the labeled protein synthesis and NO treatment periods. The enhancement of ubiquitination by GSNO treatment was not seen with the C145S hAGT mutant (which went from 12 to 14%), confirming that the formation of S-nitrosocysteine at this position is the reason for the enhanced recognition by the ubiquitination/degradation system.

The enhanced ubiquitination of hAGT by GSNO in this system is not attributable to a general increase in the activity of the ubiquitin conjugating system because it was not seen when the target protein was SSAT, which is known to be an excellent substrate for this reaction (Fig. 9; Ref. 18). In fact, when SSAT was tested, the ubiquitin conjugation level was decreased slightly from 69 to 50%.

**DISCUSSION**

The COOH-terminal His-tagged hAGT constructed for use in the current experiments is clearly superior to the recombinant protein with an NH2-terminal extension His6-tag used in the past. The lower activity of the latter may be attributable to the addition of 12 additional amino acids and/or to the presence of the His6-tag sequence in this part of the protein. The crystal structure of non-His-tagged hAGT showed that it contains a bound zinc ion that is coordinated to amino acids Cys-5, Cys-24, His-29, and His-85 in the NH2-terminal domain (30). The crystals of an NH2-terminal His-tagged hAGT did not contain this zinc atom (31). Although the function of the bound zinc is not fully understood, removal of zinc from the protein or mutation of the coordinating residues to reduce zinc occupancy reduces the rate of...
The COOH-terminal region, in which the His-tag in AGT-His6 was placed, causes a significant conformational change. The terminal His-tag, which is located very close to Cys-5, causes a significant conformational change.

Cys-145 residue quite sensitive to either direct nitrosylation or to nitrosylation via the active site. These interactions are likely to render the hAGT protein (34, 35). hAGT clearly conforms to this motif with the known interactions of Cys-145 with His-146 and Glu-172.

Although reaction of tyrosine residues in hAGT was also seen in response to exposure to SNAP, this reaction appears to occur at Tyr-68 rather than Tyr-114 or Tyr-158 and is unlikely to contribute to theloss of activity. The sensitivity of Cys-145 to reaction with NO is confirmed by the rapid inactivation of hAGT in CHO cells and the subsequent ubiquitination and degradation of the hAGT protein. Because this degradation does not occur with the C145S or C145A mutant hAGTs, it is clear that the key target of nitric oxide leading to hAGT degradation is this cysteine residue.

S-Nitrosylation reactions are potentially readily reversible, but the rapid degradation of the hAGT protein after formation of the S-nitrosyl-Cys-145 adduct would convert the inactivation into a permanent loss of DNA repair activity. The ubiquitination of hAGT after formation of S-alkyl adducts at Cys-145 during DNA repair appears to be attributable to a conformational change in the protein that allows its recognition by an E2/E3 ubiquitin ligase complex. This conformational change is seen in crystals of hAGT after reaction with either free base O6-methylguanine or O6-benzylguanine (30). It is brought about by two factors: the disruption of the hydrogen bond network involving Cys-145 described above, and the sterically driven helix displacement caused by the adducts being in close van der Waals contact with the carbonyl oxygen of Met-134 and the helix in which this residue is located. This results in an opening of the asparagine-hinge formed by residues from Asn-137 to Pro-144. It is very likely that the formation of S-nitrosocysteine at Cys-145 has a similar effect. This is therefore a highly specific and novel consequence of reaction with NO limited to hAGT. Degradation of actin was not changed by nitration. It has been reported that proteosomal degradation of p53 was reduced after nitration, leading to its accumulation (36). Also, there was an inhibition of proteasomal activity in general when macrophages were treated with GSNO or stimulated to produce NO endogenously (36). Therefore, the greatly decreased half-life of hAGT protein in the NO-treated cells occurs despite a reduction in the general rate of protein breakdown. This unique response of hAGT to exposure to SNAP, this reaction appears to occur at.

The ability of NO or species derived from it to damage DNA either directly or via the formation of intermediates such as nitrosamines, and thus initiate mutagenic and carcinogenic events, is well established (13–15). The inactivation of DNA repair processes provides an

![Figure 8: Effect of C145A mutation on hAGT degradation upon reaction with NO. CHO cells were treated with 10 mM SNAP or 0.5% DMSO (control) for 1 h, and 200 µM cycloheximide was then added to culture medium; cells were harvested at 0, 2, and 4 h later, as shown. A, levels of C145 hAGT and actin protein determined by Western blotting after separation of 20 µg of cell extract by SDS-PAGE developed with anti-AGT and anti-actin antibody. B, the percentages of C145A hAGT remaining in cell extracts normalized with respect to actin as described in Fig. 6.](image)

![Figure 9: Formation of ubiquitinated adducts in wild-type hAGT, C145S hAGT, and SSAT in reticulocyte lysates system. The hAGT, C145S hAGT, and SSAT labeled with [35S]methionine were synthesized from a coupled transcription/translation system. The proteins were exposed to 5 mM GSNO for 1 h and then incubated with a reticulocyte lysate degradation system as described in “Materials and Methods.” Aliquots were then separated by SDS-PAGE, and radioactivity in the gel was determined by PhosphorImager SI and ImageQuant software.](image)
additional way in which DNA damage may lead to neoplastic growth in cells subjected to chronic exposure to NO as a result of inflammation or infection (15, 37). Several DNA repair proteins are known to be inhibited by NO including glycosylases that rely on zinc finger motifs and DNA ligases, as well as the loss of the hAGT repair function shown here. The rapid degradation of this important DNA repair protein is of particular interest in the light of the ability of NO-generating processes to form N-nitroso compounds that are known to generate alkylation damage in DNA for which AGT is a critical repair process (6, 38, 39).

REFERENCES

Inactivation and Degradation of $O^6$-Alkyguanine-DNA Alkyltransferase after Reaction with Nitric Oxide

Liping Liu, Meng Xu-Welliver, Sreenivas Kanugula, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/11/3037

Cited articles
This article cites 37 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/11/3037.full.html#ref-list-1

Citing articles
This article has been cited by 28 HighWire-hosted articles. Access the articles at:
/content/62/11/3037.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.