Published in final edited form as:

Sci Transl Med. 2010 February 17; 2(19): 19ra13. doi:10.1126/scitranslmed.3000328.

S-nitrosylation from GSNOR Deficiency Impairs DNA Repair and Promotes Hepatocarcinogenesis

Wei Wei¹, Bin Li¹, Martha A. Hanes⁴, Sanjay Kakar², Xin Chen³, and Limin Liu^{1,*}

¹Department of Microbiology and Immunology, University of California, San Francisco, California 94143, USA.

²Department of Pathology and Veteran Affairs, University of California, San Francisco, California 94143, USA.

³Department of Biopharmaceutical Sciences, University of California, San Francisco, California 94143, USA.

⁴Department of Laboratory Animal Resources, University of Texas Health Science Center at San Antonio, Texas 78229, USA.

Abstract

Human hepatocellular carcinoma is associated with elevated expression of inducible nitric oxide synthase (iNOS), but the role of nitric oxide in the pathogenesis of hepatocellular carcinoma remains unknown. Here we show that the abundance and activity of S-nitrosoglutathione reductase (GSNOR), a protein critical for control of protein S-nitrosylation, are significantly decreased in about 50% of HCC patients. GSNOR-deficient (GSNOR^{-/-}) mice are very susceptible to spontaneous and carcinogen-induced hepatocellular carcinoma. Livers in GSNOR^{-/-} mice, during inflammatory responses, sustain substantial S-nitrosylation and proteasomal degradation of the key DNA repair protein O⁶-alkylguanine-DNA alkyltransferase. Repair of carcinogenic O⁶-alkylguanines in GSNOR^{-/-} mice is significantly impaired. Predisposition to hepatocellular carcinoma, S-nitrosylation and depletion of alkylguanine-DNA alkyltransferase, and accumulation of O⁶-alkylguanines are all abolished in GSNOR^{-/-}iNOS^{-/-} mice. Thus, GSNOR deficiency, through dysregulated S-nitrosylation, inactivates DNA repair system and promotes hepatocellular carcinoma.

*To whom correspondence should be addressed: Limin Liu, 513 Parnassus Avenue, HSE-201J, San Francisco, CA 94143, Tel: 415-476-1466, Fax: 415-502-4995, Limin.Liu@ucsf.edu.

LIST OF SUPPLEMENTARY MATERIAL

Materials and Methods

Supplemental Table 1. Clinical data of HCC patients.

Fig. S1. Human GSNOR in HCC and noncancerous liver tissues.

Fig. S2. Tumorigenesis in GSNOR^{-/-} mice.

Fig. S3. No increase in liver injury or inflammation in aged tumor-free GSNOR^{-/-} mice.

Fig. S4. No difference in proliferation index in livers of wild-type and GSNOR^{-/-} mice before HCC development.

Fig. S5. AGT activity in liver of 3-week-old resting GSNOR^{-/-} and wildtype mice.

Fig. S6. Protection of AGT from nitrosative inactivation by GSNOR autonomously in hepatocytes.

Fig. S7. Increase in AGT ubiquitination in GSNOR^{-/-} mice.

Fig. S8. AGT in human HCCs.

References

Author contributions: W.W. designed and performed experiments and analyzed data; B.L. performed experiments of clinical samples and analyzed data; M.A.H. and S.K. provided histopathologic interpretation of tissue specimens; X.C. provided clinical samples, data and expertise; L.L. designed the research, analyzed the data, and wrote the paper.

Competing interests: The authors declare no competing interests.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide (1), and HCC incidence and mortality in the United States is rapidly increasing (2). The etiology of HCC is well established, with the majority of HCC attributable to chronic hepatitis from hepatitis B or C virus infection (3). Nevertheless, the molecular mechanisms through which risk factors contribute to hepatocarcinogenesis, for the most part, remain poorly understood (4).

Inducible nitric oxide synthase, responsible for high-output production of nitric oxide in innate immune response and inflammation, is often highly increased, at mRNA and protein levels, in the hepatocytes of patients with chronic hepatitis B or C virus infection (5–7), hemochromatosis (8), and alcoholic cirrhosis (9), all of which cause predisposition to HCC. Furthermore, iNOS is expressed in the hepatocytes within HCCs (7, 10), and HCC patients exhibit elevated concentrations of plasma nitrite/nitrate (11, 12). Studies with iNOS^{-/-} mice, in spontaneous and fibrosis-associated models of HCC, revealed little effect of iNOS-derived nitric oxide on hepatocarcinogenesis (13). The amount of nitric oxide bioactivity, however, is regulated not only by nitric oxide synthases, but also by enzymatic degradation (14–16). Defective degradation can result in excessive amount of nitric oxide bioactivity in vivo (14), and whether nitric oxide plays a role in hepatocarcinogenesis remains unclear.

S-nitrosylation is a major mechanism through which nitric oxide modifies the functions of proteins to exert control over biological processes (17). Protein S-nitrosylation is thus a potential modulator of cellular processes important for tumorigenesis, including inhibition or induction of apoptotic cell death and inhibition of DNA repair (17, 18). The DNA repair enzyme O⁶-alkylguanine-DNA alkyltransferase (AGT) repairs mutagenic and cytotoxic O⁶-alkylguanines, which can be mispaired by DNA polymerases to thymine during DNA replication, causing G:C to A:T transition (19). O⁶-alkylguanines are produced by alkylating N-nitroso compounds that are present in the environment and formed endogenously through either NOS-dependent or -independent pathways (19–21). Mice deficient in AGT are more susceptible to HCC induced by dimethylnitrosamine (22), whereas overexpression of AGT in transgenic mice reduces both diethylnitrosamine-induced and spontaneous HCC (23, 24), indicating a critical protective role of AGT against HCC. AGT can be inactivated by S-nitroso-N-acetylpenicillamine and S-nitrosoglutathione (GSNO) through S-nitrosylation of the cysteine in the enzyme active site in vitro (18). Nevertheless, the role of protein S-nitrosylation in the development of HCC or any other tumor has not been directly investigated.

S-Nitrosoglutathione reductase (GSNOR) (also known as alcohol dehydrogenase class III), a ubiquitous, phylogenetically conserved enzyme, is the cell's primary means for degrading the main non-protein S-nitrosothiol (SNO), S-nitrosoglutathione (GSNO) (14, 15). GSNO is in equilibrium with protein SNOs in cells, and GSNOR controls cellular concentration of protein SNOs (14–16, 25). Mice deficient in GSNOR exhibit large increases in protein S-nitrosylation and tissue injury after iNOS induction; the protective function of GSNOR against nitrosative stress is particularly prominent in the liver (14). The human GSNOR gene (*ADH5*) is located at approximately 4q23, a region in which chromosomal deletion occurs most frequently in HCC (26–29). Furthermore, deletion in 4q23 occurs frequently in cirrhotic and dysplastic hepatocytes, the precursor cells for HCC (26, 30). The gene(s) potentially important to HCC in the region remains to be identified.

During our study of GSNOR^{-/-} mice, we noticed a high incidence of spontaneous liver tumors. Here, we have established that the protein amount and activity of GSNOR is frequently deficient in human HCC. We then employed GSNOR^{-/-} mice and found that

GSNOR is important for protection against both spontaneous and diethylnitrosamine-induced HCC. Finally, we found that GSNOR protects O⁶-alkylguanine-DNA alkyltransferase from disruptive S-nitrosylation and subsequent proteasomal degradation, thus revealing a likely molecular mechanism by which GSNOR prevents mutagenesis and carcinogenesis.

RESULTS

Frequent Deficiency of GSNOR in Human HCC

To investigate potential deficiency of GSNOR in human HCC, we first measured GSNOR activity in pairs of HCC and associated noncancerous liver tissues from 24 HCC patients (Fig. 1 and fig. S1). Whereas GSNOR activity in noncancerous liver without or with cirrhosis was similar (fig. S1A), the activity is significantly decreased in HCC, with reduction of GSNOR activity in 50% of the HCCs (Fig. 1, A and B). GSNOR activity was at least 50% lower in 10 (P1–P10) out of the 24 HCCs than in the paired noncancerous tissues, and the activity in 5 (P1–P5) of the HCCs was decreased by 80%–90% (Fig. 1A and fig. S1B). In two additional patients (P19 and P21), GSNOR activity was low in both HCC and noncancerous tissues (Fig. 1A). For the other 12 patients, GSNOR activity in HCC appeared to be normal since it did not significantly differ from the activity in associated noncancerous liver or from the group mean of the noncancerous livers (Fig. 1, A and B). Thus GSNOR activity was low in 50% of the HCC patients. To investigate whether deficiency of GSNOR activity in HCC results from reduction of GSNOR protein, we conducted quantitative immunoblot analysis of GSNOR (Fig. 1C). In pairs with relatively lower GSNOR activity in HCC, the amount of GSNOR protein was lower in HCC than in noncancerous tissue (Fig. 1C). The decrease in GSNOR activity in HCC was correlated to the decrease in GSNOR protein amount (fig. S1C). Thus, both GSNOR activity and protein amount are frequently decreased in human HCC, and reduced amount of protein is likely a main cause of decrease in GSNOR activity.

Predisposition of GSNOR^{-/-} Mice to Spontaneous HCC

To study the role of GSNOR in tumorigenesis, we macroscopically and histologically analyzed age-matched wild-type C57BL/6 (n = 53) and congenic GSNOR^{-/-} (n = 54) mice for spontaneous tumors (Fig. 2 and fig. S2). GSNOR^{-/-} mice, on the C57BL/6 genetic background known to be resistant to spontaneous hepatotumorigenesis (31), frequently developed hepatocellular adenomas (HCA) and HCCs (Fig. 2), and in one instance developed hepatoblastoma (fig. S2). Hepatocellular tumors between 5–20 mm in diameter started to appear in GSNOR^{-/-} mice at 1.5 year, and the tumor-free survival of GSNOR^{-/-} mice was significantly decreased (Fig. 2C). A GSNOR^{-/-} mouse frequently developed multiple hepatocellular tumors, often consisting of both HCA and HCC (Fig. 2 and fig. S2, A and B). Incidence of HCA and HCC in GSNOR^{-/-} mice was respectively 4- and 10-fold greater than that in wild-type controls (Fig. 2D). The incidence of hepatocellular tumor was increased in both male (Fig. 2E) and female (Fig. 2F) GSNOR^{-/-} mice, with a greater increase in males. The incidence of all types of hepatocellular tumors and of HCC in GSNOR^{-/-} males was about 50% and 30%, respectively. Thus, GSNOR^{-/-} mice are predisposed to spontaneous HCA and HCC.

Incidence of other spontaneous tumors, including those of spleen, kidney, heart and lung, was not significantly increased in GSNOR^{-/-} mice (fig. S2C). For instance, the incidence of spontaneous lymphoma, a common neoplasm in aged mice, was 20% in GSNOR^{-/-} (n = 54) and 24% in wild-type C57BL/6 (n = 45) mice. Thus the increase in spontaneous tumorigenesis in GSNOR^{-/-} mice in the experimental setting appears to be specific to the liver.

Spontaneous hepatotumorigenesis in a number of transgenic mouse models is associated with chronic liver injury and increased hepatocyte turnover (32–37). However, serum concentrations of alanine aminotransferase, a marker of liver injury, did not differ between tumor-free GSNOR^{-/-} mice and wild-type controls (fig. S3A) (14). Liver histology of GSNOR^{-/-} mice, except for an increase in hepatocellular tumors, was indistinguishable from that of age-matched wild-type controls and showed no sign of increased inflammation (fig. S3, B and C). In addition, immunohistochemical staining for Ki67, a marker of proliferating cells, showed no difference between the livers of GSNOR^{-/-} mice and wild-type controls prior to HCC development (fig. S4). Thus, GSNOR^{-/-} mice do not appear to suffer chronic liver injury and compensatory regeneration.

Increase in Spontaneous Hepatocarcinogenesis in GSNOR^{-/-} Mice is Abolished by Genetic Deletion of iNOS

To determine whether iNOS activity plays a role in spontaneous hepatocarcinogenesis in GSNOR^{-/-} mice, we studied the incidence of hepatocellular tumors in GSNOR^{-/-}iNOS^{-/-} double-knockout mice. GSNOR^{-/-}iNOS^{-/-} mice, congenic to C57BL/6, develop and reproduce normally. The high incidence of HCA and HCC in GSNOR^{-/-} males was reduced in age-matched GSNOR^{-/-} iNOS^{-/-} males to that of wild-type controls (Fig. 3). Thus, nitric oxide bioactivity, likely SNO from iNOS, predisposes liver to spontaneous HCC in the absence of GSNOR, and protection against the hepatocarcinogenic activity of iNOS requires a physiological function of GSNOR.

GSNOR^{-/-} Mice Are More Susceptible to Chemical Hepatocarcinogenesis

To further study the role of GSNOR in hepatocarcinogenesis, we employed GSNOR^{-/-} mice in a diethylnitrosamine (DEN)-induced HCC model (38). DEN, one of a group of alkylating N-nitroso compounds both present in the environment and formed endogenously (20, 21, 39), is a genotoxic compound that causes tumorigenesis predominantly in liver (39). We treated 15-day-old male mice with a single intraperitoneal injection of DEN (5 µg/g) and analyzed DEN-induced tumor formation at the age of 10.5 months, when spontaneous liver tumors do not occur. The DEN-treated GSNOR^{-/-} mice developed over 5-fold more liver tumors than the wild-type controls (Fig. 4, A to C). Many of the tumors in a DEN-treated GSNOR^{-/-} mouse were much larger than the ones in a wild-type control (Fig. 4, A and B), and the maximal tumor diameters in GSNOR^{-/-} mice were more than 4 fold greater than those of wild-type controls (Fig. 4D). The increase in tumor multiplicity and maximal size in GSNOR^{-/-} mice was abolished in GSNOR^{-/-} iNOS^{-/-} mice (Fig. 4, C and D). Thus loss of GSNOR, likely through inability to control iNOS-derived SNO, significantly promotes DEN-induced hepatocarcinogenesis.

DEN-challenged GSNOR^{-/-} Mice Exhibit Marked Decrease in O⁶-alkylguanine-DNA Alkyltransferase

GSNOR could potentially promote breakdown of mutagenic N-nitroso compounds through glutathione-mediated denitrosation. However, DEN metabolism does not involve glutathione-mediated denitrosation (39); consequently, GSNOR is not expected to promote direct denitrosation and inactivation of DEN. DEN, after being activated in hepatocytes, causes a potent mutagenic and carcinogenic DNA lesion, O⁶-ethylguanine (39). DEN-induced hepatocarcinogenesis is significantly inhibited by overexpression of the DNA repair enzyme AGT (23). To investigate the potential effect of GSNOR on AGT, we measured AGT activity in mouse livers. The amount of liver AGT activity was not different between resting GSNOR^{-/-} and wild-type mice (fig. S5). Strikingly, however, 6 days after a single intraperitoneal injection of DEN, the liver AGT activity in GSNOR^{-/-} mice was about 80% lower than in the wild-type control (Fig. 5A). Liver lactate dehydrogenase activity, on the other hand, did not differ among the DEN-challenged mice (Fig. 5B). Thus, in response to

DEN challenge, liver AGT activity was selectively decreased in GSNOR^{-/-} mice. DEN causes liver inflammation (40), and one major inflammatory mediator is iNOS. The amount of liver AGT activity was not lower in DEN-challenged GSNOR^{-/-}iNOS^{-/-} mice than in the wild-type control (Fig. 5A). Thus, AGT activity in the DEN-challenged GSNOR^{-/-} mouse is likely decreased by SNO from iNOS, and GSNOR is required to protect AGT from nitrosative inactivation.

We carried out immunoblot analysis with an antibody to mouse AGT and found that after DEN challenge, the concentration of AGT protein in the liver of GSNOR^{-/-} mice was much lower than that in wild-type and GSNOR^{-/-}iNOS^{-/-} mice (Fig. 5C). The AGT protein concentration did not differ among all the unchallenged mice. These data indicate that the SNO-dependent decrease in AGT activity in DEN-challenged GSNOR^{-/-} mice results from reduction of AGT protein.

Inhibition of DNA Repair in DEN-challenged GSNOR^{-/-} Mice

To determine whether nitrosative inactivation of AGT is associated with impaired repair of carcinogenic O⁶-alkylguanines, we analyzed genomic DNA from livers of DEN-challenged mice by immuno-slot blot with a monoclonal antibody against O⁶-ethyldeoxyguanosine (41). While no O⁶-ethyldeoxyguanosine was detected without DEN challenge, at 2 days after DEN injection, substantial amount of O⁶-ethyldeoxyguanosine was detected and the concentrations of O⁶-ethyldeoxyguanosine were equivalent in wild-type, GSNOR^{-/-}, and GSNOR^{-/-}iNOS^{-/-} mice (Fig. 6). Whereas O⁶-ethyldeoxyguanosine, as expected, was mostly repaired by day 6 after DEN injection in wildtype mice, the amount of O⁶-ethyldeoxyguanosine at this time was higher in GSNOR^{-/-} mice. Furthermore, repair of O⁶-ethyldeoxyguanosine was restored in GSNOR^{-/-}iNOS^{-/-} mice. Thus, repair of carcinogenic O⁶-alkylguanine is impaired by nitrosative stress in GSNOR^{-/-} mice. In addition, the concentration of O²-ethyldeoxythymidine, the long-lasting DNA lesion from DEN that is not repaired by AGT (39), was comparable among DEN-treated wild-type, GSNOR^{-/-}, and GSNOR^{-/-}iNOS^{-/-} mice (Fig. 6), suggesting that the effect of GSNOR deficiency on DNA lesion appears to be specific to O⁶-alkylguanine.

Depletion of AGT through Proteasomal Degradation in GSNOR^{-/-} Mice during Inflammatory Responses

To examine protection of AGT from nitrosative inactivation by GSNOR as a general mechanism during inflammatory responses, we studied AGT in livers of mice challenged with an intraperitoneal injection of lipopolysaccharide (LPS), which causes systematic inflammation and iNOS expression in hepatocytes (14, 42). Whereas the LPS challenge had little effect on liver AGT protein abundance in wild-type mice, the treatment resulted in almost complete loss of AGT protein in GSNOR^{-/-} (Fig. 7A). Loss of AGT after LPS challenge was prevented in GSNOR^{-/-}iNOS^{-/-} mice (Fig. 7A). Thus, GSNOR is required to protect AGT from nitrosative inactivation in the LPS model. Furthermore, AGT in hepatocytes isolated from GSNOR^{-/-} mice was more susceptible to nitrosative inactivation than that in wildtype hepatocytes (fig. S6), indicating protection of AGT by GSNOR in the cells autonomously. Thus, AGT in liver cells appears to be generally highly susceptible to nitrosative stress and critically depends on GSNOR for protection.

Intact AGT protein is quite stable, but after inactivation the protein is rapidly degraded through the ubiquitin-proteasome pathway (18, 43). To investigate the role of proteasomal degradation in the control of AGT protein abundance in GSNOR^{-/-} mice during inflammatory responses, LPS-challenged mice at 2 days after LPS injection were given an intraperitoneal injection of MG262, a specific inhibitor of the proteasome (44). Although treatment with MG262 for ~4 h had little effect on liver AGT concentration in wild-type and

GSNOR^{-/-}iNOS^{-/-} mice, the treatment in GSNOR^{-/-} mice brought the AGT protein concentration largely back to that of resting mice (Fig. 7A). Similarly, in the DEN model, MG262 treatment, equivalent to that described above, largely prevented decrease in AGT in GSNOR^{-/-} mice (Fig. 5D). In addition, following inhibition of the proteasome by the MG262 treatment in DEN-challenged mice, we detected more polyubiquitinated AGT in GSNOR^{-/-} than in wild-type and GSNOR^{-/-}iNOS^{-/-} mice (fig. S7). Thus the SNO-dependent decrease in AGT during inflammatory responses in GSNOR^{-/-} mice is brought about by proteasomal degradation of AGT, presumably damaged AGT.

S-nitrosylation of AGT in Liver of GSNOR^{-/-} Mice

AGT is susceptible to *S*-nitrosylation and inactivation by GSNO in vitro (18). We found that *S*-nitrosylated AGT formed by treatment with *S*-nitroso-cysteine can be specifically detected by the biotin switch method (45) in conjunction with an anti-AGT antibody (Fig. 7B). This SNO assay does not recognize AGT in the liver lysate treated with other redox chemicals, including hydrogen peroxide (Fig. 7B). When proteasomal degradation of AGT was inhibited by MG262, we detected AGT *S*-nitrosylation that had been formed endogenously in the livers of LPS-challenged GSNOR^{-/-} mice (Fig. 7C). The concentration of *S*-nitrosylated AGT was higher in GSNOR^{-/-} than in wild-type control, and AGT SNO was abolished in GSNOR^{-/-}iNOS^{-/-} mice (Fig. 7C). AGT is thus a direct target of *S*-nitrosylation in vivo, and GSNOR is required to prevent abnormally elevated *S*-nitrosylation of AGT from iNOS during inflammatory responses.

DISCUSSION

GSNOR Deficiency in Human HCC

We found in this study that the amount of GSNOR in HCC in about 50% of patients is decreased by 50% to 90%. This common deficiency of GSNOR is consistent with recurrent chromosomal deletion in human HCC at 4q22-23, which contains the human GSNOR gene (26–29). In addition to loss of one of the two alleles of the GSNOR gene, however, there may be other defects (genetic or epigenetic) that contribute to the 80–90% reduction in GSNOR activity in about 20% of HCCs. Normal GSNOR activity in HCCs in 50% of the patients suggests that hepatocyte dedifferentiation during HCC development is unlikely by itself to cause GSNOR deficiency. It remains to be investigated whether reduction of GSNOR activity in noncancerous liver, as in patients P19 and P21, might predispose to HCC. Because GSNOR prevents *S*-nitrosylation-mediated degradation of the key DNA repair protein AGT and protects against hepatocarcinogenesis in mice, we suggest that a deficiency of GSNOR with concurrent overexpression of iNOS in the development of human HCC (5–7, 9) results in dysregulated *S*-nitrosylation that is likely to be a common contributing factor in human HCC. Indeed, gene-expression profiling of liver tissue adjacent to HCC showed that both GSNOR deficiency and iNOS overexpression are closely associated with a poor prognosis in HCC patients (46).

Protection of Mice from HCC by GSNOR

A principal discovery of this study is that GSNOR, the key protein in the control of *S*-nitrosothiols, is critically important for protection against hepatocarcinogenesis in animals. GSNOR^{-/-} mice were more susceptible to both spontaneous and carcinogen-induced HCC. Predisposition of GSNOR^{-/-} mice to HCC was abolished by genetic deletion of iNOS. Thus GSNOR protects mice from HCC, most likely through its physiological action on *S*-nitrosothiols. This conclusion is further supported by the observation that GSNOR is required to prevent iNOS-dependent *S*-nitrosylation and depletion of the key DNA repair enzyme AGT in inflammatory responses.

Before the appearance of spontaneous HCC, the mice that are derived from targeted deletion of the genes, *mdr2* (35), *Acox1* (33), *Pten* (34), *Nrf1* (36), *Sod1* (32), and *Nemo* (37), sustain chronic liver injury and consequent repeated rounds of hepatocyte death and regeneration, a process involving repeated rounds of DNA replication under inflammation that is thought to increase DNA mutation and hepatocarcinogenesis (32–37). Mice transgenic for the core gene of hepatitis C virus, on the other hand, develop HCC spontaneously at old age in the absence of necroinflammation (47). Similarly, *GSNOR*^{-/-} mice do not appear to suffer chronic liver injury. Thus spontaneous hepatocarcinogenesis in *GSNOR*^{-/-} mice may not result from chronic liver injury and hepatocyte turnover that is common in most other transgenic models of spontaneous HCC.

Our study has also revealed a pro-hepatocarcinogenic effect of iNOS in the absence of *GSNOR*. iNOS is constitutively expressed in ileal epithelium of normal mice (48), and helps to prevent opportunistic infection by commensal gastrointestinal microorganisms in liver and other organs in mice reared under specific-pathogen-free (not germ-free) conditions (49). Thus, even in the absence of chronic inflammation, liver cells in our mice may be exposed to reactive nitrogen species from iNOS that is constitutively expressed or induced in response to commensal microorganisms. *GSNOR* deficiency results in altered response to iNOS activation, including nitrosative inactivation of AGT, and consequently makes the mice more susceptible to nitrosative stress. iNOS is expressed in hepatocytes under conditions that predispose to HCC and in HCC in both animals and humans (5–7, 13, 50). Studies with *iNOS*^{-/-} mice in spontaneous and fibrosis-associated models of HCC, nevertheless, revealed little effect of iNOS on hepatocarcinogenesis (13, 51). Our results suggest that the potential pro-hepatocarcinogenic activity of iNOS is normally prevented or masked by *GSNOR*. Induction of iNOS (52) and loss of chromosome 4q (53) occur in lung, breast, and other cancers. Thus *GSNOR* deficiency and protein S-nitrosylation might also contribute to the development of other cancers.

Protection of AGT from S-nitrosylation and Depletion by *GSNOR*

Our results suggest that the increased susceptibility to spontaneous and DEN-induced HCC in *GSNOR*^{-/-} mice may result from S-nitrosylation and depletion of AGT during inflammatory responses. We showed previously that the concentration of liver protein SNOs, derived from iNOS activity, increases greatly during inflammation in *GSNOR*^{-/-} mice (14). Our data show that one of the proteins highly susceptible to S-nitrosylation by iNOS is AGT and that protection of AGT from S-nitrosylation requires *GSNOR*. In vitro recombinant human AGT is susceptible to S-nitrosylation at the enzyme active site Cys145 (18), where both strong nucleophilicity of the cysteine sulfur and the close proximity of His146 (54) may promote S-nitrosylation (17). S-nitrosylation of Cys145, which likely causes conformational changes in AGT (54), appears to be responsible for rapid proteasomal degradation of AGT when CHO cells with transfected human AGT are treated with S-nitroso-N-acetylpenicillamine (18). S-nitrosylation of AGT likely results in rapid degradation and loss of AGT in *GSNOR*^{-/-} mice. The enzyme active site Cys149 of mouse AGT, with its nucleophilic sulfur and conserved His150 in close proximity, is probably the site of S-nitrosylation in vivo. Depletion of liver AGT in response to both LPS and DEN challenges in *GSNOR*^{-/-} mice, but not in *GSNOR*^{-/-}*iNOS*^{-/-} mice, suggests that AGT is generally highly susceptible to nitrosative inactivation in inflammatory responses. Our results, taken together, establish a critical role for *GSNOR* in protection of AGT from hyper-S-nitrosylation and proteasomal degradation. In a few human HCC samples that are deficient in *GSNOR* activity, AGT protein amount is decreased (fig. S8), suggesting possible protection of AGT by *GSNOR* in human.

Extensive DNA mutations affecting multiple cellular pathways are the hallmark of carcinogenesis (55). We found that nitrosative inactivation of AGT, the protein critical for

repair of carcinogenic O⁶-alkylguanines, impairs repair of O⁶-ethylguanine in the liver of GSNOR^{-/-} mouse, thus revealing a possible mechanism of hepatocarcinogenesis from GSNOR deficiency. Unrepaired O⁶-ethylguanine is mispaired to thymine during DNA replication and causes G:C to A:T transition in the next round of DNA replication. G:C to A:T transition, which may arise from either O⁶-alkylguanines or deamination of 5-methylcytosine at CpG sites, is the most common mutation in cancer (55, 56). Accumulation of O⁶-alkylguanines from nitrosative inactivation of AGT likely increases DNA mutation and, through mutations of oncogenes and tumor suppressor genes, could promote initiation of DEN-induced hepatocarcinogenesis in GSNOR^{-/-} mice. AGT overexpression in transgenic mice decreases the frequency of G:C to A:T mutations and reduces spontaneous HCC, suggesting that spontaneous O⁶-alkylguanine lesions can occur also (24). Although significant increase in spontaneous HCC is not detected in AGT-deficient mice up to about 10 months of age (22), the effect of AGT deficiency on spontaneous HCC in old, close to 2-year-old mice is unknown. Loss of AGT is associated with G:C to A:T mutation of key oncogenes and tumor suppressor genes in a number of human cancers (19). We thus suggest that nitrosative inactivation of AGT in inflammatory responses may allow mutagenesis from spontaneous O⁶-alkylguanine lesions and contribute significantly to spontaneous HCC in GSNOR^{-/-} mice. In addition to nitrosative inactivation of AGT, iNOS-derived nitric oxide bioactivity may contribute to the formation of alkylating N-nitroso compounds and thus DNA mutation in hepatocarcinogenesis (20). Dysregulated S-nitrosylation from GSNOR deficiency may also accelerate HCC development, possibly through promoting survival or growth of neoplastic cells (52, 57, 58).

Our results underscore the importance of protein S-nitrosylation in the pathogenesis of HCC. Thus patients with GSNOR deficiency and concurrent iNOS overexpression in the liver may be at a significantly increased risk of HCC, and inhibition of iNOS-derived S-nitrosylation in these patients may provide a therapeutic strategy to prevent HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S.-T. Cheung and S. T. Fan at the University of Hong Kong for providing the human liver samples; UCSF Liver Center/SF VAMC pathology core, M. Foster, H. Willenbring, and M. Egeblad for technical assistance; L. Coussens, J. Stamler, Z. Werb, and B. Yen for reading the manuscript and comments.

Funding: Supported by the Sandler Family Supporting Foundation, Stewart Trust Cancer Research Award, the UCSF Liver Center Grant P30 DK26743, UC Cancer Research Coordinating Committee Research Grant, and NIH CA122359 (L.L.).

REFERENCES AND NOTES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005; 55:74–108. [PubMed: 15761078]
2. El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. *Ann Intern Med.* 2003; 139:817–823. [PubMed: 14623619]
3. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol.* 2006; 45:529–538. [PubMed: 16879891]
4. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer.* 2006; 6:674–687. [PubMed: 16929323]

5. Majano PL, Garcia-Monzon C, Lopez-Cabrera M, Lara-Pezzi E, Fernandez-Ruiz E, Garcia-Iglesias C, Borque MJ, Moreno-Otero R. Inducible nitric oxide synthase expression in chronic viral hepatitis. Evidence for a virus-induced gene upregulation. *J Clin Invest*. 1998; 101:1343–1352. [PubMed: 9525976]
6. Kane JM 3rd, Shears LL 2nd, Hierholzer C, Ambs S, Billiar TR, Posner MC. Chronic hepatitis C virus infection in humans: induction of hepatic nitric oxide synthase and proposed mechanisms for carcinogenesis. *J Surg Res*. 1997; 69:321–324. [PubMed: 9224400]
7. Rahman MA, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H, Ono T, Yamanoi A, Kohno H, Nagasue N. Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. *Clin Cancer Res*. 2001; 7:1325–1332. [PubMed: 11350902]
8. Hussain SP, Raja K, Amstad PA, Sawyer M, Trudel LJ, Wogan GN, Hofseth LJ, Shields PG, Billiar TR, Trautwein C, Hohler T, Galle PR, Phillips DH, Markin R, Marrogi AJ, Harris CC. Increased p53 mutation load in nontumorous human liver of wilson disease and hemochromatosis: oxyradical overload diseases. *Proc Natl Acad Sci U S A*. 2000; 97:12770–12775. [PubMed: 11050162]
9. McNaughton L, Puttagunta L, Martinez-Cuesta MA, Kneteman N, Mayers I, Moqbel R, Hamid Q, Radomski MW. Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc Natl Acad Sci U S A*. 2002; 99:17161–17166. [PubMed: 12482944]
10. Ikeguchi M, Ueta T, Yamane Y, Hirooka Y, Kaibara N. Inducible nitric oxide synthase and survivin messenger RNA expression in hepatocellular carcinoma. *Clin Cancer Res*. 2002; 8:3131–3136. [PubMed: 12374680]
11. Moussa YI, Plevris JN, Hayes PC. Plasma nitrites/nitrates in HCV infection and hepatocellular carcinoma. *Eur J Gastroenterol Hepatol*. 2000; 12:159–163. [PubMed: 10741929]
12. Moriyama A, Masumoto A, Nanri H, Tabaru A, Unoki H, Imoto I, Ikeda M, Otsuki M. High plasma concentrations of nitrite/nitrate in patients with hepatocellular carcinoma. *Am J Gastroenterol*. 1997; 92:1520–1523. [PubMed: 9317076]
13. Denda A, Kitayama W, Kishida H, Murata N, Tamura K, Kusuoka O, Tsutsumi M, Nishikawa F, Kita E, Nakae D, Konishi Y, Kuniyasu H. Expression of inducible nitric oxide (NO) synthase but not prevention by its gene ablation of hepatocarcinogenesis with fibrosis caused by a choline-deficient, L-amino acid-defined diet in rats and mice. *Nitric Oxide*. 2007; 16:164–176. [PubMed: 16931074]
14. Liu L, Yan Y, Zeng M, Zhang J, Hanes MA, Ahearn G, McMahon TJ, Dickfeld T, Marshall HE, Que LG, Stamler JS. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell*. 2004; 116:617–628. [PubMed: 14980227]
15. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature*. 2001; 410:490–494. [PubMed: 11260719]
16. Que LG, Liu L, Yan Y, Whitehead GS, Gavett SH, Schwartz DA, Stamler JS. Protection from Experimental Asthma by an Endogenous Bronchodilator. *Science*. 2005; 308:1618–1621. [PubMed: 15919956]
17. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*. 2005; 6:150–166. [PubMed: 15688001]
18. Liu L, Xu-Welliver M, Kanugula S, Pegg AE. Inactivation and degradation of O(6)-alkylguanine-DNA alkyltransferase after reaction with nitric oxide. *Cancer Res*. 2002; 62:3037–3043. [PubMed: 12036910]
19. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer*. 2004; 4:296–307. [PubMed: 15057289]
20. Liu RH, Baldwin B, Tennant BC, Hotchkiss JH. Elevated formation of nitrate and N-nitrosodimethylamine in woodchucks (*Marmota monax*) associated with chronic woodchuck hepatitis virus infection. *Cancer Res*. 1991; 51:3925–3929. [PubMed: 1855209]
21. Lundberg JO, Weitzberg E, Cole JA, Benjamin N. Nitrate, bacteria and human health. *Nat Rev Microbiol*. 2004; 2:593–602. [PubMed: 15197394]
22. Iwakuma T, Sakumi K, Nakatsuru Y, Kawate H, Igarashi H, Shiraishi A, Tsuzuki T, Ishikawa T, Sekiguchi M. High incidence of nitrosamine-induced tumorigenesis in mice lacking DNA repair methyltransferase. *Carcinogenesis*. 1997; 18:1631–1635. [PubMed: 9276640]

23. Nakatsuru Y, Matsukuma S, Nemoto N, Sugano H, Sekiguchi M, Ishikawa T. O6-methylguanine-DNA methyltransferase protects against nitrosamine-induced hepatocarcinogenesis. *Proc Natl Acad Sci U S A*. 1993; 90:6468–6472. [PubMed: 8341657]
24. Zhou ZQ, Manguino D, Kewitt K, Intano GW, McMahan CA, Herbert DC, Hanes M, Reddick R, Ikeno Y, Walter CA. Spontaneous hepatocellular carcinoma is reduced in transgenic mice overexpressing human O6- methylguanine-DNA methyltransferase. *Proc Natl Acad Sci U S A*. 2001; 98:12566–12571. [PubMed: 11606727]
25. Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ. A central role for S-nitrosothiols in plant disease resistance. *Proc Natl Acad Sci U S A*. 2005; 102:8054–8059. [PubMed: 15911759]
26. Yeh SH, Chen PJ, Shau WY, Chen YW, Lee PH, Chen JT, Chen DS. Chromosomal allelic imbalance evolving from liver cirrhosis to hepatocellular carcinoma. *Gastroenterology*. 2001; 121:699–709. [PubMed: 11522754]
27. Patil MA, Gutgemann I, Zhang J, Ho C, Cheung ST, Ginzinger D, Li R, Dykema KJ, So S, Fan ST, Kakar S, Furge KA, Buttner R, Chen X. Array-based comparative genomic hybridization reveals recurrent chromosomal aberrations and JAB1 as a potential target for 8q gain in hepatocellular carcinoma. *Carcinogenesis*. 2005
28. Wong N, Lai P, Lee SW, Fan S, Pang E, Liew CT, Sheng Z, Lau JW, Johnson PJ. Assessment of genetic changes in hepatocellular carcinoma by comparative genomic hybridization analysis: relationship to disease stage, tumor size, and cirrhosis. *Am J Pathol*. 1999; 154:37–43. [PubMed: 9916916]
29. Nagai H, Pineau P, Tiollais P, Buendia MA, Dejean A. Comprehensive allelotyping of human hepatocellular carcinoma. *Oncogene*. 1997; 14:2927–2933. [PubMed: 9205099]
30. Raidl M, Pirker C, Schulte-Hermann R, Aubele M, Kandioler-Eckersberger D, Wrba F, Micksche M, Berger W, Grasl-Kraupp B. Multiple chromosomal abnormalities in human liver (pre)neoplasia. *J Hepatol*. 2004; 40:660–668. [PubMed: 15030983]
31. Turturro A, Duffy P, Hass B, Kodell R, Hart R. Survival characteristics and age-adjusted disease incidences in C57BL/6 mice fed a commonly used cereal-based diet modulated by dietary restriction. *J Gerontol A Biol Sci Med Sci*. 2002; 57:B379–B389. [PubMed: 12403793]
32. Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ, Huang TT. CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene*. 2005; 24:367–380. [PubMed: 15531919]
33. Fan CY, Pan J, Usuda N, Yeldandi AV, Rao MS, Reddy JK. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J Biol Chem*. 1998; 273:15639–15645. [PubMed: 9624157]
34. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, Mizuno K, Hasegawa G, Kishimoto H, Iizuka M, Naito M, Enomoto K, Watanabe S, Mak TW, Nakano T. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest*. 2004; 113:1774–1783. [PubMed: 15199412]
35. Mauad TH, van Nieuwkerk CM, Dingemans KP, Smit JJ, Schinkel AH, Notenboom RG, van den Bergh Weerman MA, Verkruijsen RP, Groen AK, Oude Elferink RP, et al. Mice with homozygous disruption of the *mdr2* P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis. *Am J Pathol*. 1994; 145:1237–1245. [PubMed: 7977654]
36. Xu Z, Chen L, Leung L, Yen TS, Lee C, Chan JY. Liver-specific inactivation of the *Nrf1* gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. *Proc Natl Acad Sci U S A*. 2005; 102:4120–4125. [PubMed: 15738389]
37. Luedde T, Beraza N, Kotsikoris V, van Loo G, Nenci A, De Vos R, Roskams T, Trautwein C, Pasparakis M. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell*. 2007; 11:119–132. [PubMed: 17292824]
38. Vesselinovitch SD, Mihailovich N. Kinetics of diethylnitrosamine hepatocarcinogenesis in the infant mouse. *Cancer Res*. 1983; 43:4253–4259. [PubMed: 6871863]

39. Verna L, Whysner J, Williams GM. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacol Ther.* 1996; 71:57–81. [PubMed: 8910949]
40. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKK β couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell.* 2005; 121:977–990. [PubMed: 15989949]
41. Nehls P, Adamkiewicz J, Rajewsky MF. Immuno-slot-blot: a highly sensitive immunoassay for the quantitation of carcinogen-modified nucleosides in DNA. *J Cancer Res Clin Oncol.* 1984; 108:23–29. [PubMed: 6746711]
42. Knowles RG, Merrett M, Salter M, Moncada S. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem J.* 1990; 270:833–836. [PubMed: 1700698]
43. Srivenugopal KS, Yuan XH, Friedman HS, Ali-Osman F. Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry.* 1996; 35:1328–1334. [PubMed: 8573590]
44. Kisselev AF, Goldberg AL. Proteasome inhibitors: from research tools to drug candidates. *Chem Biol.* 2001; 8:739–758. [PubMed: 11514224]
45. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol.* 2001; 3:193–197. [PubMed: 11175752]
46. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM, Golub TR. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med.* 2008; 359:1995–2004. [PubMed: 18923165]
47. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med.* 1998; 4:1065–1067. [PubMed: 9734402]
48. Hoffman RA, Zhang G, Nussler NC, Gleixner SL, Ford HR, Simmons RL, Watkins SC. Constitutive expression of inducible nitric oxide synthase in the mouse ileal mucosa. *Am J Physiol.* 1997; 272:G383–G392. [PubMed: 9124364]
49. Shiloh MU, MacMicking JD, Nicholson S, Brause JE, Potter S, Marino M, Fang F, Dinanuer M, Nathan C. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity.* 1999; 10:29–38. [PubMed: 10023768]
50. Ahn B, Han BS, Kim DJ, Ohshima H. Immunohistochemical localization of inducible nitric oxide synthase and 3-nitrotyrosine in rat liver tumors induced by N-nitrosodiethylamine. *Carcinogenesis.* 1999; 20:1337–1344. [PubMed: 10383909]
51. Tatemichi M, Tazawa H, Masuda M, Saleem M, Wada S, Donehower LA, Ohgaki H, Ohshima H. Suppression of thymic lymphomas and increased nonthymic lymphomagenesis in Trp53-deficient mice lacking inducible nitric oxide synthase gene. *Int J Cancer.* 2004; 111:819–828. [PubMed: 15300793]
52. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. *Nat Rev Cancer.* 2006; 6:521–534. [PubMed: 16794635]
53. Struski S, Doco-Fenzy M, Cornillet-Lefebvre P. Compilation of published comparative genomic hybridization studies. *Cancer Genet Cytogenet.* 2002; 135:63–90. [PubMed: 12072205]
54. Daniels DS, Mol CD, Arvai AS, Kanugula S, Pegg AE, Tainer JA. Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical base binding. *Embo J.* 2000; 19:1719–1730. [PubMed: 10747039]
55. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA. Human cancers express a mutator phenotype. *Proc Natl Acad Sci U S A.* 2006; 103:18238–18242. [PubMed: 17108085]
56. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE,

- Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006; 314:268–274. [PubMed: 16959974]
57. Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem*. 1997; 272:31138–31148. [PubMed: 9388267]
58. Liu L, Stamler JS. NO: an inhibitor of cell death. *Cell Death Differ*. 1999; 6:937–942. [PubMed: 10556969]

Fig. 1. GSNOR is frequently deficient in human HCC. **(A)** GSNOR activity in HCC (red) and associated noncancerous liver (green) tissues from 24 patients. P, patient. Data are from 2–4 independent experiments with the mean indicated by the bar. **(B)** The GSNOR activity in the HCCs is significantly lower than that in the noncancerous liver tissues in the 24 patients ($P = 0.01$; Wilcoxon rank-sum test). **(C)** Western blot analysis of GSNOR. NT, non-tumor liver tissue; HCC, cancer tissue.

Fig. 2. GSNOR^{-/-} mice are predisposed to spontaneous hepatocellular tumors. **(A)** Liver of a GSNOR^{-/-} mouse with two tumors (arrows). **(B)** Hematoxylin and eosin (H&E)-stained section of HCC from a GSNOR^{-/-} mouse. Scale bar, 100 μm. **(C)** Hepatocellular (HC) tumor-free survival curves from Kaplan-Meier analysis. Wild-type (WT) (n = 53) versus GSNOR^{-/-} (n = 54), P = 0.007 by Log-rank test. **(D, E and F)** Incidence of HCC, HCA, and all hepatocellular tumors (All) in all the mice **(D)**, males **(E)** (26 WT, 29 GSNOR^{-/-}), and females **(F)** (27 WT, 25 GSNOR^{-/-}). Tumor incidence is shown by a percentage of mice with tumor. Significant differences between wild-type and age-matched GSNOR^{-/-} mice are indicated by one (P < 0.05) or two (P < 0.005) asterisks.

Fig. 3.

Spontaneous hepatocellular tumors in the GSNOR^{-/-} background is decreased by deletion of iNOS. GSNOR^{-/-}iNOS^{-/-} (n = 28), GSNOR^{-/-} (n = 29), and wild-type (n = 26) males were analyzed histopathologically. Tumor incidence is shown by a percentage of mice with tumor. Incidence of all hepatocellular tumors (P = 0.007) and of HCC alone (P = 0.013) in GSNOR^{-/-}iNOS^{-/-} mice was significantly lower than in GSNOR^{-/-} mice.

Fig. 4. Diethylnitrosamine-induced tumor development is increased in $GSNOR^{-/-}$ mice. (**A** and **B**) Representative livers of male wild-type and $GSNOR^{-/-}$ mice 10 months after DEN ($5 \mu\text{g/g}$) injection. (**C**) Number of tumors ($\geq 1 \text{ mm}$) per mouse and (**D**) maximal tumor diameters (mean $\pm$ SE) in DEN-treated wild-type (WT) ($n = 13$), $GSNOR^{-/-}$ (KO) ($n = 10$), and $GSNOR^{-/-}iNOS^{-/-}$ (DKO) ($n = 14$) mice. $GSNOR^{-/-}$ mice develop significantly more tumors than wild-type ($P < 0.001$) and $GSNOR^{-/-}iNOS^{-/-}$ ($P < 0.001$) mice; the maximal tumor size in $GSNOR^{-/-}$ is significantly bigger than that in wild-type ($P < 0.001$) and $GSNOR^{-/-}iNOS^{-/-}$ ($P = 0.003$).

Fig. 5.

O⁶-alkylguanine-DNA alkyltransferase is depleted in livers of DEN-challenged GSNOR^{-/-} mice. **(A)** AGT activity in liver lysates of mice 6 days after DEN (37.5 μg/g) injection. Data are mean (± SE) from 4 wild-type (WT), 5 GSNOR^{-/-} (KO), and 3 GSNOR^{-/-}iNOS^{-/-} (DKO) mice. The AGT activity in GSNOR^{-/-} mice is significantly lower than that in wild-type ($P < 0.004$) and GSNOR^{-/-}iNOS^{-/-} ($P < 0.001$) mice. **(B)** Lactate dehydrogenase (LDH) activity in liver lysates of the DEN-challenged mice described in (A). Data are mean (± SD) from 4 wild-type, 3 GSNOR^{-/-}, and 3 GSNOR^{-/-}iNOS^{-/-} mice. **(C)** Immunoblot of AGT, GSNOR, and β-actin in livers of wild-type, GSNOR^{-/-}, and GSNOR^{-/-}iNOS^{-/-} mice before or 6 days after DEN injection. The amount of liver lysate in the last lane, 7.5 μg, is 30% of that in the other lanes. **(D)** Immunoblot of AGT and GAPDH in livers of mice 4 days after treatment with DEN or DEN followed by the proteasome inhibitor MG262.

Fig. 6. Repair of O⁶-ethyldeoxyguanosine in livers of GSNOR^{-/-} mice is impaired. Immuno-slot blot of genomic DNA from livers of wild-type (WT), GSNOR^{-/-} (KO), and GSNOR^{-/-}iNOS^{-/-} (DKO) mice before (-DEN) or 2 and 6 days after DEN injection. The DNA (1 ug per slot) is probed with monoclonal antibodies against O⁶-ethyldeoxyguanosine (O⁶-ethyl-dG) and O²-ethyldeoxythymidine (O²-ethyl-dT), respectively.

Fig. 7.

S-nitrosylation and inactivation of AGT in livers of GSNOR^{-/-} mice. **(A)** Immunoblot of AGT and β -actin in livers of wild-type (WT), GSNOR^{-/-} (KO), and GSNOR^{-/-}iNOS^{-/-} (DKO) mice treated with no LPS (-LPS), LPS (+LPS), or LPS followed by the proteasome inhibitor MG262 (+LPS +MG262). **(B)** Detection of *S*-nitrosylated AGT (SNO AGT) by the biotin switch assay and AGT immunoblot. Samples were GSNOR^{-/-}iNOS^{-/-} liver lysate treated with no chemical (-), 300 μ M cysteine (Cys), 300 μ M *S*-nitroso-cysteine (SNO), 1 mM dithiothreitol (DTT), or 300 μ M hydrogen peroxide (H₂O₂). (Bottom row) Immunoblot of total AGT in each sample. **(C)** Biotin switch analysis of endogenously *S*-nitrosylated AGT in livers of LPS-challenged mice. MG262 was injected into some of the mice to inhibit proteasomal degradation of AGT. (Bottom row) Total AGT.