

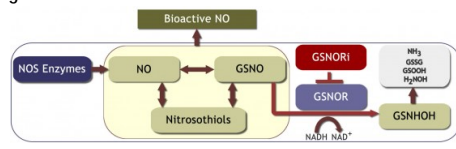
Abstract

S-nitrosoglutathione reductase (GSNOR) is the primary enzyme responsible for the metabolism of S-nitrosoglutathione (GSNO). Through this denitrosylation process, GSNOR plays a central role in regulating the levels of endogenous S-nitrosothiols and protein S-nitrosylation-based signalling. Recent evidence suggests that modulation of proteins via S-nitrosylation may alter DNA repair mechanisms. For example, the DNA repair enzyme O⁶-alkylguanine-DNA alkyltransferase (AGT) can be inactivated through S-nitrosylation of the Cys in the active site of AGT. AGT corrects O⁶-alkylguanines and thus prevents the mispairing of cytotoxic O⁶-alkylguanines to thymine by DNA polymerases during DNA replication. Mice deficient in AGT have been shown to be more susceptible to hepatocarcinogenesis induced by dimethylnitrosamine. Previous studies have shown that total genetic deletion of GSNOR in mice (GSNOR^{-/-}) leads to a higher incidence of hepatocellular carcinoma possibly through uncontrolled iNOS-induced nitrosylation and subsequent proteasomal degradation of AGT. These mice also demonstrate reduced AGT activity in livers following diethylnitrosamine (DEN) challenge. We sought in the current study to investigate whether mice with heterozygous deletion of GSNOR (GSNOR^{+/-}) are protected from nitrosative inactivation of AGT and hepatocarcinogenesis. GSNOR^{+/-} mice, produced by crossing GSNOR^{-/-} mice with wildtype C57BL/6 mice, were given a single intraperitoneal injection of DEN. Mice were sacrificed either 6 days or 10 months after DEN injection to study AGT protein expression and tumor development in the liver. In the DEN-treated GSNOR^{+/-} mice, AGT levels were equivalent to that seen in wild-type control animals and no increased incidence of hepatocellular tumors was evident. The findings indicate that, in contrast to complete genetic deletion of GSNOR, GSNOR protein remaining after a 50% reduction from mono-allelic deletion in GSNOR^{+/-} mice appears sufficient to maintain normal AGT activity and protect against hepatocarcinogenesis.

Introduction

GSNO is one of the primary endogenous sources of bioavailable NO (Figure 1). GSNO and NO concentrations are regulated by GSNOR reductase (GSNOR)^{1,2} which plays critical roles in many biological systems. Studies of germline deletion of the GSNOR gene in mice leads to DEN-induced hepatocellular carcinoma through S-nitrosylation and proteasomal degradation of the key DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (AGT)³. Additional work by Wei et al. with targeted deletion of GSNOR in hepatocytes of mice found that during inflammatory responses induced by IP injection of DEN or lipopolysaccharide, the amount of liver AGT was almost completely depleted⁴. We sought in the current study to investigate whether mice with heterozygous deletion of GSNOR (GSNOR^{+/-}) are protected from nitrosative inactivation of AGT and hepatocarcinogenesis.

Figure 1: Schematic of the Role of GSNO/GSNOR



Methods

Animals

GSNOR^{-/-} mice (3), which had been backcrossed 10 times to C57BL/6, were bred with C57BL/6 wild-type mice to obtain GSNOR^{+/-} mice. All mice were maintained on normal mouse chow (5058 PicoLab Mouse Diet 20) in a specific pathogen-free facility at the UCSF. Analysis by polymerase chain reaction of liver samples from three GSNOR^{-/-} mice found that they were not infected by Helicobacter (UC Davis Comparative Pathology Laboratory). The experimental protocol was approved by the Institutional Animal Care and Use Committee of UCSF.

DEN Acute Effects and Hepatocarcinogenesis

Mice were given at day 15 a single intraperitoneal injection of DEN in PBS (50 µg/g) to study acute toxicity. Body weights were measured on Day 6 (FIGURE 2). Mice were sacrificed 6 days after DEN injection. Liver samples were collected and flash frozen in liquid nitrogen.

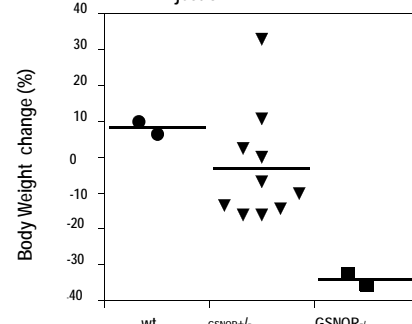
Mice were given at day 15 a single intraperitoneal injection of DEN in PBS (5 µg/g) to induce chemical hepatocarcinogenesis. These mice were held for 10 months and then sacrificed. At necropsy, externally visible tumors (>1 mm in diameter) in the livers were counted and measured under stereomicroscopy (FIGURES 5A & 5B).

GSNOR and AGT Protein Expression

Proteins in liver homogenates from the 6 days of DEN exposure were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with rabbit antiserum to GSNOR, β-actin mouse monoclonal antibody (A-5441; Sigma), or goat antiserum to AGT (R&D Systems). GSNOR, β-actin, and AGT were detected and quantified with infrared fluorescent secondary antibodies—a goat antibody to rabbit coupled to Alexa Fluor 680 (Molecular Probes), a goat antibody to mouse coupled to IRDye 800 (Rockland Immunochemicals), and a donkey antibody to goat coupled to Alexa Fluor 680 (Molecular Probes)—with an infrared fluorescence imaging system (Odyssey; LICOR Biosciences) FIGURE 3&4.

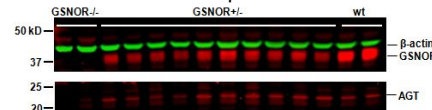
Results

FIGURE 2 Body Weight Change in Mice 6 Days after DEN Injection



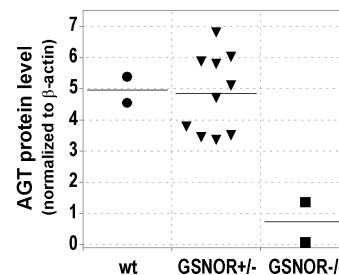
Results

FIGURE 3. Response to DEN Challenge: AGT and GSNOR Protein Expression



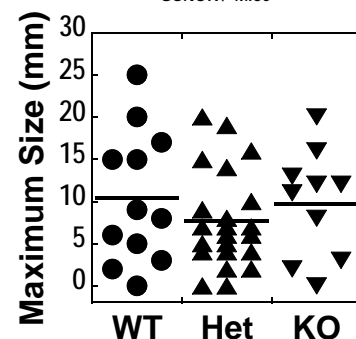
Immunoblot detection of AGT, GSNOR and β-actin in liver lysate (37.5µg) 6 days after DEN injection

FIGURE 4. AGT Protein is Equivalent in Wildtype and Heterozygous GSNOR Mice, but Reduced in GSNOR KO Mice



AGT protein amounts were normalized to β-actin in each sample. The proteins were detected and quantified with infrared fluorescent secondary antibodies using an infrared fluorescence imaging system

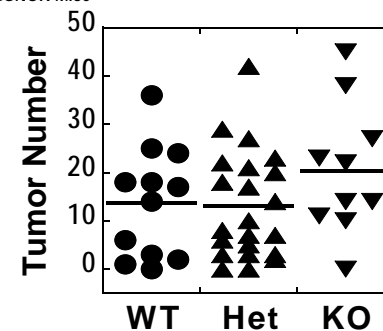
Figure 5A. Tumor Sizes from Wild-type, GSNOR^{+/-} and GSNOR^{-/-} Mice



Maximal tumor diameters in wild-type (n=12), GSNOR^{+/-} (Het) (n=22), and GSNOR^{-/-} (KO) (n=10) 10 months after DEN injection

Results

FIGURE 5B. Tumor Numbers in WT, Heterozygous and KO GSNOR Mice



Number of tumors (>0.5mm) per mouse in wild-type (WT) (n=12), GSNOR^{+/-} (Het) (n=22), and GSNOR^{-/-} (KO) (n=10) months after DEN (5µg/g) injection

Conclusions

- ❑ In contrast to GSNOR^{-/-} knock out mice, animals heterozygous for GSNOR^{+/-} have AGT levels comparable to wild type mice
- ❑ The body weights of GSNOR^{+/-} mice were similar to those of wild type mice, in contrast to the KO mice that lost substantially more weight following DEN administration
- ❑ No increases in tumor size or frequency were evident in the heterozygous GSNOR^{+/-} mice compared to wildtype mice.
- ❑ Partial genetic deletion of GSNOR is not associated with adverse sequelae in the liver

Literature Cited

- 1) 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
- 2) 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(5728):1618–1621.
- 3) Wei W, Li B, Hanes M, Kakr S, Chen X, and L Liu L. S-nitrosylation from GSNOR Deficiency Impairs DNA Repair and Promotes Hepatocarcinogenesis. *Sci Trans Med*. 2010 ;2 (19) 1-9.
- 4) Wei W, Yang Z, Tang CH and Liu L Targeted deletion of GSNOR in hepatocytes of mice causes nitrosative inactivation of O⁶-alkylguanine-DNA alkyltransferase and increased sensitivity to genotoxic diethylnitrosamine. *Carcinogenesis*. 2011; doi: 10.1093/carcin/bgr041