AKR1A1 is a novel mammalian S-nitroso-glutathione reductase

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Running title: Novel GSNO reductase

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Abstract

Oxidative modification of Cys residues by nitric oxide (NO) results in S-nitrosylation, a ubiquitous posttranslational modification and a primary mediator of redox-based cellular signaling. Steady-state levels of S-nitrosylated proteins are largely determined by denitrosylase enzymes that couple NAD(P)H oxidation with reduction of S-nitrosothiols, including protein and low-molecular-weight (LMW) S-nitrosothiols (S-nitroso-glutathione (GSNO) and S-nitroso-coenzyme A (SNO-CoA)). SNO-CoA reductases (SCoRs) require NADPH, whereas enzymatic reduction of GSNO can involve either NADH or NADPH. Notably, GSNOR (Adh5) accounts for most NADH-dependent GSNO reductase activity, while NADPH-dependent GSNO reductase activity is largely unaccounted for (CBR1 mediates a minor portion). Here, we de novo purified NADPH-coupled GSNO reductase activity from mammalian tissues and identified aldo-keto reductase family 1 member A1 (AKR1A1), the archetypal mammalian SCoR, as a primary mediator of NADPH-coupled GSNO reductase activity in these tissues. Kinetic analyses suggested an AKR1A1 substrate preference of SNO-CoA > GSNO. AKR1A1 deletion from murine tissues dramatically lowered NADPH-dependent GSNO reductase activity. Conversely, GSNOR-deficient mice had increased AKR1A1 activity, revealing potential cross-talk among GSNO-dependent denitrosylases. Molecular modeling and mutagenesis of AKR1A1 identified Arg-312 as a key residue mediating the specific interaction with GSNO; in contrast, substitution of the SNO-CoA–binding residue Lys-127 minimally affected the GSNO-reducing activity of AKR1A1. Together, these findings indicate that AKR1A1 is a multi-LMW-SNO reductase that can distinguish between and metabolize the two major LMW-SNO signaling molecules GSNO and SNO-CoA, allowing for wide-ranging control of protein S-nitrosylation under both physiological and pathological conditions.

Physiological nitric oxide (NO)-based signaling is transduced primarily by S-nitrosylation, the phylogenetically-conserved posttranslational modification of Cys thiols by NO to form S-nitrosothiol (SNOs) (1), with broad-ranging effects across health and disease (2, 3). Both the formation of S-nitroso-proteins (SNO-proteins) and the removal of NO from SNO-proteins are governed enzymatically. SNO formation entails the combined actions of nitric
oxide synthases, SNO synthases, and trans-nitrosylases (4), whereas denitrosylase enzymes terminate SNO-based signals. The activity of denitrosylases generally determines the steady-state levels of cellular S-nitrosylation (5).

Low-molecular-weight (LMW) thiols that carry SNO, including S-nitroso-glutathione (GSNO) and S-nitroso-coenzyme A (SNO-CoA), are central to protein S-nitrosylation through their ability to establish S-nitrosylation/denitrosylation equilibria with target SNO-proteins (5). Denitrosylation reactions involving LMW-SNO are generally coupled to the oxidation of NAD(P)H. In particular, SNO-CoA reductases (SCoRs) in yeast (Adh6) and mammals (AKR1A1) oxidize NADPH to reduce SNO-CoA, thereby regulating SNO-protein levels (6, 7); no appreciable NADH-dependent SCoR activity has been identified to-date (6). By contrast, GSNO reductase (GSNOR; Adh5) metabolizes GSNO using reducing equivalents from NADH (8, 9). Such GSNOR-dependent regulation of S-nitrosylation widely impacts cellular function in both health and disease (5). However, while NADH/NAD⁺ ratios are often << 1, the ratio of NADPH/NADP⁺ is typically >>1; NADPH therefore serves as the general reducing agent in cells (5). Evidence for NADPH-coupled reduction of GSNO was found in the enzyme carbonyl reductase 1 (CBR1) (10); however, it is unclear to what extent CBR1 operates across cells and tissues. Therefore, we sought to identify additional enzymes by performing de novo purification and characterization of NADPH-dependent GSNO reductase activity from mammalian tissues.

Results

**NADPH-dependent GSNO reductase activity is present across tissues**

NADPH oxidation in the presence of GSNO was observed to varying degrees in lysates from mouse tissues (Fig. 1A), with the highest activity found in the kidneys. Previous reports suggested that the thioredoxin/thioredoxin reductase (Trx/TrxR) system can metabolize GSNO using NADPH as reducing equivalent (11); however, addition of the TrxR inhibitor auranofin to mouse tissue extracts had no effect on NADPH oxidation in the presence of GSNO (Fig. 1A). Further, GSNOR-deficiency increased GSNO-dependent NADPH oxidation in both kidneys and liver, but not in the lungs, heart, or spleen (Fig. 1A). This suggests a compensatory increase in NADPH-dependent GSNOR activity when NADH-dependent GSNOR activity is lost, particularly in tissues with high GSNOR activity (Fig. 1B). Acute treatment of kidney extracts from GSNOR⁻/⁻ mice with GSNO increased SNO-protein levels (Fig. 1C, lanes 3 and 4), which was reversed by the co-addition of NADPH (Fig. 1C, lanes 5 and 6). Treatment with auranofin did not prevent NADPH-dependent protein denitrosylation (Fig. 1C, lane 5 vs lane 6), indicating that neither Trx/TrxR-mediated protein denitrosylation (12) nor GSNO metabolism are dominant in this system. Together, these results identify NADPH-dependent GSNOR reductase activity across tissues to regulate protein S-nitrosylation.

**Purification and identification of AKR1A1 as novel NADPH-coupled GSNO reductase activity in mammalian tissues**

NADPH-dependent GSNO reductase activity was purified to homogeneity from bovine kidney extract (Fig. 2, Fig. S1, and Table S1) and identified as aldo-keto reductase 1A1 (AKR1A1). AKR1A1 is the founding member of the aldo-keto reductase superfamily of oxidoreductases, and orthologs are found throughout the vertebrate subphylum (13). Importantly, we previously identified AKR1A1 as the primary mammalian NADPH-dependent SNO-CoA reductase (6), and have described a renoprotective role for AKR1A1-dependent denitrosylation via metabolic reprogramming following acute kidney injury (7). Immunodepletion of AKR1A1 from kidney extracts of wild-type (C57Bl/6) mice (Fig. 3A) markedly reduced NADPH-dependent GSNO reductase activity (Fig. 3B). Further, NADPH-dependent GSNO reductase activity is reduced by ~50% in kidney extracts from AKR1A1⁻⁻ mice and ~90% in kidney extracts from AKR1A1⁻⁻ mice (Fig. 3, C and D). Finally, kidney extracts from AKR1A1⁺⁺ and AKR1A1⁻⁻ mice were treated with GSNO alone or together with NADPH, and total SNO-protein levels were assessed by SNO-RAC with Coomassie blue...
staining (Fig. 3E). Treatment with GSNO increased SNO-protein levels in both AKR1A1+/+ and AKR1A1-/- kidney extracts (Fig. 3E, lanes 3 and 4). SNO-protein levels were drastically reduced by co-addition of NADPH with GSNO in AKR1A1+/+ kidney extracts (Fig. 3E, lane 5), but not in AKR1A1-/- extracts (Fig. 3E, lane 6). Together, these data confirm that AKR1A1 is responsible for the majority of NADPH-dependent GSNO reductase activity in kidney tissues.

AKR1A1-dependent GSNO reductase activity was assessed across multiple tissues: namely the liver, lungs, heart, and spleen (Fig. 3, F and G). AKR1A1 accounts for a significant portion of NADPH-dependent GSNO reductase activity in the liver, lungs, and spleen, but not in the heart (Fig. 3F), corresponding to the relative expression of AKR1A1 in these tissues (Fig. 3G). This suggests that the role of AKR1A1 as a GSNO reductase will be most prominent in tissues with higher expression levels (7), and also that additional reductases may be present to account for remaining activity in AKR1A1-/- tissues.

As demonstrated in Figure 1A, NADPH-dependent GSNO reductase activity is increased in both the kidneys and liver from GSNOR-/- mice. To determine whether AKR1A1 could account for this increased GSNO metabolizing activity, we performed western blot analysis for AKR1A1 in liver extracts from GSNOR+/+ and GSNOR-/- mice. AKR1A1 protein level was increased in liver extract from GSNOR-/- mice (Fig. 3, H and I), suggesting that AKR1A1 expression is upregulated in the absence of GSNOR, the principal NADH-dependent GSNO reductase, and providing evidence for a physiological role of AKR1A1-mediated GSNO reduction. Additionally, quantitative RT-PCR across a variety of tissues (Fig. S2) suggests that basal AKR1A1 activity may be higher than that of either GSNOR (Adh5) or CBR1; however, definitive analyses require mRNA copy numbers and enzyme activity measurements. Taken together, these data indicate that AKR1A1 likely serves as a physiological GSNO reductase across tissues.

Enzymatic properties of AKR1A1 using GSNO as substrate

Product analysis by MS revealed glutathione sulfinamide (Fig. S3) to be the major product of GSNO reduction by AKR1A1 in the presence of NADPH, suggesting a hydride transfer reaction mechanism (Fig. 4A) similar to both the NADPH-dependent reduction of SNO-CoA by AKR1A1 (6, 14) and the NADH-dependent reduction of GSNO by GSNO R (8, 15). The hydride transfer mechanism common to aldo-keto reductases requires an active site tyrosine (Tyr50 in AKR1A1) to act as a proton donor for the reaction (16). To confirm the requirement of this residue for the reduction of GSNO by AKR1A1, we generated and purified catalytically-dead AKR1A1Y50A and assessed its ability to reduce GSNO. Reaction of AKR1A1Y50A with GSNO failed to consume NADPH, compared to AKR1A1 WT (Fig. 4B). Together these data confirm that reduction of GSNO by AKR1A1 follows the canonical AKR reaction scheme, specifically hydride transfer from NADPH to the N-atom of the SNO moiety and protonation of the O-atom by the active site Tyr50 to generate the S-(N-hydroxy) intermediate that rearranges to form glutathione sulfinamide (8). Serial addition of limiting amounts of NADPH in the presence of excess GSNO demonstrated a 1:1 GSNO:NADPH stoichiometry (Fig. 4C). Kinetic analysis of purified bovine AKR1A1 revealed $K_m$ of 87.8 ± 5.1 µM and $K_{cat}$ of 471 ± 8 min⁻¹ (Fig. 4D), while analysis of purified recombinant human AKR1A1 gave $K_m$ of 184.1 ± 8.1 µM and $K_{cat}$ of 948 ± 14 min⁻¹ (Fig. 4E).

The results presented thus far identify AKR1A1 as a major NADPH-dependent GSNO reductase in mammalian tissues; AKR1A1 is also the major SNO-CoA reductase across tissues (6). Thus, AKR1A1 represents the first LMW-SNO reductase that metabolizes two major SNO signaling molecules. We sought to further understand the basis of molecular recognition of GSNO vs. SNO-CoA by AKR1A1 (14). Modeling of GSNO with the active site of AKR1A1 produced a catalytically competent binding mode with the S—N—O group oriented towards the catalytic Tyr50 and cofactor NADPH (Fig. 5A). The active site of AKR1A1 is surrounded by multiple charged and aromatic amino acids (Fig. 5A, highlighted in...
Novel GSNO reductase

red) that could mediate the GSNO—AKR1A1 interaction, and the predicted binding mode identified Trp22 and Arg312 as potential mediators of this interaction. In order to test the role of these amino acids and other active site residues in binding of GSNO, we generated and purified AKR1A1 mutants (14) and tested the effect of mutation on in vitro reduction of GSNO. Mutations of AKR1A1 had varying effects on the catalytic efficiency of AKR1A1 for GSNO (Fig. 5B, Table 2, and Table S2), with some mutations increasing catalytic efficiency but others reducing catalytic efficiency. Most notably, AKR1A1R312A drastically reduced catalytic efficiency for GSNO (Fig. 5B). Arg312 was predicted to form a hydrogen bond with the α-carboxylic acid group of the glutamic acid component of GSNO (Fig. 5A). Accordingly, mutation of Arg312 to alanine led to an ~5-fold increase in Km (Fig. 5C and Table 2) suggesting that binding of GSNO is dramatically altered in this mutant. Catalytic efficiency was also reduced due to an ~60% reduction in Kcat (Fig. 5C and Table 2). In contrast, AKR1A1R312A increases Km for SNO-CoA by ~2-fold, indicating a degree of specificity in the Arg312—GSNO interaction (Table 2) (14). We previously identified Lys127 as the key residue mediating the SNO-CoA—AKR1A1 interaction (14), where mutation of this residue to alanine increases Km for SNO-CoA ~8-fold and reduces the ability of AKR1A1 to bind SNO-CoA-bound beads. Lys127 was not predicted to interact with GSNO (Fig. 5A) and accordingly, AKR1A1K127A only very slightly increased Km for GSNO (Fig. 5D and Table 2) with no effect on Kcat. These results indicate that different residues are driving the interaction between AKR1A1 and GSNO versus SNO-CoA, with Arg312 mediating the GSNO—AKR1A1 interaction and Lys127 mediating the SNO-CoA—AKR1A1 interaction.

The SNO moiety gives selective preference for AKR1A1 to interact with SNO-CoA versus reduced or derivatized CoA (14), so that the SNO-CoA reductase activity of AKR1A1 is not inhibited by cytosolic concentrations of free CoA or acetyl-CoA. We tested whether this principle applied to the interaction of GSNO with AKR1A1. Competitive inhibition assays using increasing concentrations of GSH or CoA with fixed (0.1 mM) GSNO demonstrated that GSH and CoA are weak inhibitors of GSNO reduction by AKR1A1. GSH was unable to reach 50% inhibition even at supraphysiological concentrations, whereas CoA had an IC50 of ~6.5 mM (Fig. 5E). These results again suggest that molecular recognition of SNO substrates by enzymes is facilitated by both the R group (GSH or CoA) and the SNO moiety, creating strong preference for the SNO-modified substrate.

Discussion

GSNO and SNO-CoA play central roles in cellular signaling by stabilizing NO bioactivity and targeting proteins for S-nitrosylation (5). Accordingly, the enzymatic denitrosylases controlling GSNO and SNO-CoA levels are paramount to proper regulation of SNO-based signaling. Here we used de novo purification to identify AKR1A1 as a novel NADPH-dependent GSNO reductase, responsible for this activity in kidneys, liver, lungs, and spleen; AKR1A1-mediated GSNO reductase activity is also likely to play an important role in other tissues where AKR1A1 is highly expressed, including brain, intestines, stomach, testes, and white adipose tissue (7). With the discovery of AKR1A1, there are now three identified GSNO reductases (GSNORs 1-3): NADH-dependent GSNOR (Adh5), NADPH-dependent CBR1 (GSNOR2), and NADPH-dependent AKR1A1/SCoR (GSNOR3); together, these enzymes likely account for a majority of GSNO reductase activity across tissues.

AKR1A1 and CBR1 provide the first example of mammalian denitrosylases sharing substrate (GSNO) and co-factor (NADPH). Human AKR1A1 has a Km of 184 µM and Kcat of 948 min⁻¹ for GSNO; CBR1 has a Km of 30 µM and Kcat of 450 min⁻¹ for GSNO (10), giving CBR1 an ~3-fold higher catalytic efficiency (Kcat/Km) for GSNO. Differences in tissue expression (Fig. S2), response to cellular stimuli, and regulation by posttranslational modification are likely to determine which enzyme predominates in a given tissue, compartment and condition. Careful comparisons of the SNO-proteomes regulated by each enzyme, particularly in tissues where all three GSNORs are expressed (e.g. liver, kidney, lungs, etc.) may
help delineate shared vs. specialized roles of these enzymes in regulating S-nitrosylation.

The ability of AKR1A1/SCoR to reduce GSNO identifies this enzyme with activity towards two major SNO signaling molecules (GSNO and SNO-CoA). Human AKR1A1 metabolizes SNO-CoA with a $K_m$ of 58 µM and $K_{cat}$ of 959 min$^{-1}$ (14) (Table 2); it metabolizes GSNO with a $K_m$ of 184 µM and $K_{cat}$ of 948 min$^{-1}$. Thus, AKR1A1 has an ~3-fold higher catalytic efficiency ($K_{cat}/K_m$) for SNO-CoA driven by an ~3-fold lower $K_m$ for SNO-CoA, identifying SNO-CoA as the preferred SNO substrate for AKR1A1. However, upregulation of AKR1A1 upon genetic deletion of GSNOR from mouse liver indicates that the GSNO reductase function of AKR1A1 is likely operative endogenously. More generally, although cytosolic CoA concentrations are lower than GSH levels, we do not believe thiol concentrations are limiting in formation of GSNO vs. SNO-CoA, or that absolute GSNO and SNO-CoA concentrations, per se, represent primary determinants of SNO-protein levels. Rather, we favor a model where GSH/GSNO and CoA/SNO-CoA may sub-compartmentalize, and/or AKR1A1 may interact with proteins that preferentially bind GSNO or SNO-CoA to generate layers of specificity. Identifying proteins whose SNO levels are regulated by the distinct SNO reductase functions of AKR1A1 vs. alternative denitrosylases will help clarify these issues, and represent areas of ongoing work. Thus, proteins regulated by the GSNO reductase activity of AKR1A1 may be found by comparing the SNO proteomes and interactomes of AKR1A1 with those of GSNOR and CBR1. Further, comparison of the SNO-proteome controlled by the SNO-CoA-binding mutant AKR1A1$^{K127A}$ with the SNO-proteome controlled by AKR1A1$^{WT}$ would be informative.

The inability of GSH and CoA to effectively inhibit GSNO reduction by AKR1A1 are consistent with our previous findings with regards to the inability of CoA and acetyl-CoA to inhibit SNO-CoA reduction by this enzyme (14) and further support molecular recognition of the SNO group by target enzymes. This provides enzymes with a means to selectively interact with SNO substrates within the cellular milieu where GSNO and SNO-CoA are likely minor populations compared to GSH and CoA (and other derivatives thereof). Whether this concept applies more generally to other low molecular weight-SNO denitrosylases or to protein denitrosylases such as thioredoxin-related proteins remains to be explored. However, it is most likely that both nitrosylases and denitrosylases exploit recognition of the stable SNO moiety to propagate and regulate SNO-based signaling broadly.
Experimental Procedures

NADPH-dependent GSNO reductase activity in murine tissues

Tissues (kidney, liver, lung, heart and spleen) were harvested from 10 to 12 week-old C57BL/6 or AKR1A1+/+ mice and GSNO−/− or AKR1A1−/−. Mice were used under a protocol consistent with the Guide for the Care and Use of Laboratory Animals (17) and approved by the CWRU IACUC. Tissues were homogenized in lysis buffer (50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 0.1mM EDTA, 0.1 mM DTPA, 1mM PMSF and cocktail of protease inhibitors (Roche)). The extracts were subsequently clarified by centrifugation (twice at 20,000 g at 4°C for 45 minutes), and the protein concentration was determined using the BCA assay. Activities were measured as described previously (6). Briefly, enzyme assays were performed in 50 mM phosphate buffer, pH 7.0 (containing 0.1 mM EDTA and DTPA) and contained 0.2 mM GSNO and 0.1 mM NADPH or NADH. The reactions were initiated by the addition of lysate and allowed to proceed for 1 minute. All assays were performed in duplicates or triplicates using 1-cm-path length cuvettes, and the slope was used to calculate the activity. For some samples, lysates were treated with 2 µM auranofin (prepared in DMSO) prior to performing the assays to rule out the NADPH-dependent GSNO breakdown mediated by the thioredoxin system.

Purification of NADPH-dependent GSNO reductase activity

Bovine kidneys were obtained from Rockland Immunochemicals Inc. and classified as waste (no IACUC approval required). Bovine kidney tissue (~80 g) was suspended in 100 ml of lysis buffer (50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 1mM PMSF and protease inhibitor cocktail table (Roche)). Initial lysis was performed in a blender using short pulses (5 to 6 times), followed by homogenization with 20 to 30 strokes with a Dounce homogenizer (Wheaton). Following centrifugation twice at 60,000 g for 45 min, the supernatant was taken as the starting material for assessment of enrichment of GSNO metabolizing activity. At this and all subsequent stages, enzyme activity was assessed with 0.2 mM GSNO, 0.1 mM NADPH in 50 mM phosphate buffer (pH 7) containing 0.1 mM EDTA and DTPA. The supernatant was precipitated with 30% ammonium sulfate followed by centrifugation at 20,000 g, and the resultant supernatant was re-precipitated with 60% ammonium sulfate and pelleted at 20,000 g. The second pellet was re-suspended and dialyzed against Tris buffer, pH 8.0 at 4 °C. The dialyzed extract was applied at 1ml/min onto a HiPrep Phenyl FF 16/10 column equilibrated with 20 mM Tris buffer, pH 8.0. The NADPH-dependent GSNO reductase activity was eluted with a linear 0 to 0.3 M NaCl gradient in 20 mM Tris buffer, pH 8.0. Active fractions were pooled and ammonium sulfate added to a final concentration of 1M. Sample was then loaded onto a HiTrap Phenyl HP column equilibrated with 20 mM Tris, pH 8.0 containing 1M ammonium sulfate. Elution was achieved using a linear gradient of 0.7 to 0.4M ammonium sulfate in Tris, pH 8.0 buffer. Active fractions were pooled and ammonium sulfate added to final concentration of 1M. Sample was then loaded onto a HiPrep Phenyl FF 16/10 column equilibrated with 20 mM Tris, pH 8.0 containing 1M ammonium sulfate at a flow rate of 0.5 ml/min. Finally, active fractions were concentrated to less than 200 µl volume and then loaded onto a Superdex 200 Increase 10/300 column. Purification was assessed by running the various protein fractions on an SDS-PAGE gel and visualized by Brilliant Blue-G Colloidal Stain (Sigma). Protein bands were identified using matrix-assisted laser desorption/ionization time of-flight/time-of-flight (MALDI-TOF/TOF) tandem mass spectrometry (MS), internally calibrated with trypsin autoproteolysis peaks. The MS spectrum was searched against the National Center for Biotechnology Information (NCBI) database.
using the on-line version of Protein Prospector (http://prospector.ucsf.edu/). MS analysis was carried out at Michael Hooker Proteomics and Mass Spectrometry Facility, University of North Carolina.

**Western blot analysis**

Western blot analyses were performed using standard methods (7, 14). Antibodies used were: AKR1A1 (Abnova, H00010327-D01P for western blotting and immunoprecipitation) or AKR1A1 (Proteintech, 15054-1-AP for western blotting); β-actin (Sigma-Aldrich, A1978); GSNO (Proteintech, 11051-1-AP); p97 (Fitzgerald, 10R-P104A).

**Kinetic analysis of bovine and recombinant AKR1A1**

Recombinant wild-type and mutant human AKR1A1 was purified as previously described (14). Enzyme assays were performed in 50 mM sodium phosphate (pH 7.0) containing 100 µM EDTA and DTPA, 100 µM NADPH, and varying concentrations of GSNO. GSNO was prepared freshly by reacting equal volumes of 0.1 M GSH and 0.1 M NaNO₂ in MilliQ water with 0.1 M EDTA and 0.1 M DTPA. Reactions were performed in triplicate. Initial rates were calculated from the absorbance decrease (340 nm) using a combined extinction coefficient of 7.06 mm⁻¹ cm⁻¹ for GSNO and NADPH. Kinetic parameters (Kₘ and Vₘₐₓ) were determined in GraphPad Prism 7, and Kₘ was calculated from Vₘₐₓ and enzyme concentration. For NADPH consumption curves, reactions (100 µM GSNO, 100 µM NADPH, and 20 nM recombinant human AKR1A1) were monitored for 10 minutes while measuring absorbance at 340 nm. GSH and CoA competitive inhibition assays were performed in duplicate in the presence of varying concentrations of GSH or CoA (dissolved in 50 mM sodium phosphate buffer (pH 7.0) with 100 µM EDTA/DTPA) with a static concentration of 100 µM GSNO and 20 nM recombinant human AKR1A1. IC₅₀ was determined using GraphPad Prism 7.

**AKR1A1 immunodepletion**

For immunodepletion, 10 µg of AKR1A1 Ab or control IgG Ab was bound to protein G-Sepharose beads (Amersham) in dilution buffer (50 mM phosphate buffer, pH, 7.0, 10 mM NaCl, 0.1 mM EDTA and DTPA) and the volume brought to 1ml. Coupling of the antibody to the beads was done at 4°C for 1 hr on an end-to-end rotator. Antibody-bound beads were washed 3 times with dilution buffer to remove unbound antibodies. Freshly prepared kidney extract (65 µg) from C57B/6 mice (Jackson laboratories) was added to the beads and the volume brought to 1ml with dilution buffer, followed by constant rotation overnight at 4°C. Supernatants were removed and used for *in vitro* assays and western blotting. 125 µl of supernatant were used to assay for NADPH-dependent GSNO reductase activity, as described above. To assess immunodepletion, a small volume of supernatant (25 µl) was combined with 10 µl of 4x LDS sample buffer (Invitrogen). To the beads, 10 µl of dye was added and the eluant was used as IP control.

**Denitrosylation assay**

Kidney tissues were homogenized in lysis buffer (50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTPA, 1 mM PMSF and cocktail of protease inhibitor (Roche)). The extract was centrifuged twice at 20,000g at 4°C for 45 minutes. Supernatant containing 1 mg protein was diluted in the assay buffer (50 mM phosphate buffer, pH 7.0) to achieve a final volume of 2 ml. The samples were then treated with 100 µM GSNO alone or in combination with NADPH or NADH for 10 minutes at room temperature. Reactions were stopped by addition of 3 volumes (6 ml) of ice-cold acetone, and denitrosylation was detected by SNO-RAC technique (18). In brief, acetone precipitated protein samples were dissolved in 2 ml of blocking buffer (0.2% S-methylmethanethiosulfonate (MMTS) and 2.5% SDS in HEN buffer, pH 8.0) and incubated in dark for 20 minutes at 55°C with frequent vortexing. Proteins were then precipitated with 3 volumes of ice-cold acetone at -20°C for 20 minutes. Residual MMTS was removed by resuspending the pellets in 2 ml of HENS buffer (HEN containing 1 % SDS), followed by protein precipitation with ice-cold acetone (as described in the previous step). Protein pellets were finally re-suspended in 2 ml of HENS buffer and freshly prepared beads (50 µl) and ascorbate
(final concentration 30 mM) were added. After incubation for 3.5 hours, beads were subsequently washed 4 times with HENS buffer and 2 times with HENS/10 (1:10 dilution of HEN buffer containing 1% SDS). Proteins were eluted with 20 µl of elution buffer (HENS/10 containing 10% β-mercaptoethanol) with constant shaking for 20 minutes at room temperature. Eluted samples were mixed with sample buffer, separated by SDS-PAGE, and visualized by Coomassie blue staining.

**Products of AKR1A1-catalyzed GSNO catabolism**

Samples (1ml) were prepared containing 20 mM ammonium bicarbonate buffer, 200 µM GSNO and 200 µM NADPH at 25°C. Reactions were initiated by the addition of purified bovine AKR1A1 and allowed to continue until absorbance at 340 nm indicated complete consumption of GSNO (~1 h). Samples were then centrifuged through a 10 kDa cut-off ultrafiltration membrane, and the filtrate was stored at -80°C until analyzed. For mass spectrometry analysis, samples were diluted 1:2 in HPLC grade acetonitrile. Formic acid was added to the samples at a final concentration of 0.1% (v:v). Samples were injected into a Thermo LTQ Oribtrap XL at a flow rate of 1 uL/min. The ion at 339 m/z was isolated and fragmented using CID with a normalized collision energy of 35 V and an isolation width of 3.0.

**Quantitative real-time PCR analysis of GSNO reductases**

Various mouse tissues (~20 mg) were harvested from C57BL/6J mice and preserved in RNA Later stabilization reagent (Qiagen). Tissues were homogenized in PureZOL (BioRad) on a Tissue Lyser (Qiagen) using stainless steel beads (Qiagen). Chloroform was used to extract the aqueous phase and RNA from this phase was purified using the Aurum purification kit (BioRad) following manufacturer’s instructions. For reverse transcriptase reaction, 1 µg of total RNA was transcribed to cDNA using iScriptTM Reverse Transcription Supermix (Biorad). qPCR was performed with the TaqMan method (using the Roche Universal Probe Library System) on an Applied Biosystems Step One Plus Real-Time PCR System. Relative expression of AKR1A1 versus ADH5 or CBR1 was calculated using the 2^-ΔΔCt method with normalization to 18S rRNA. For murine AKR1A1, probe no. 99 (Roche) was used with the following primers: 5’- GGTATATTTGCCCATGATTACG- 3’ and 3’- GGGAGTAGCAGGCAATG- 5’. For murine ADH5, probe no. 88 was used with the following primers: 5’- ACAGGACGCACATGGAAAG-3’ and 3’- ACACACCTTGGGACACTC-5’. For murine CBR1, probe no. 17 was used with the following primers: 5’- AGGTGACAATGAAAACGAACTTT-3’ and 3’- GGACTTAATCAACGCAAGC-5’. For murine 18S rRNA, probe no. 48 was used with the following primers: 5’- GCAATTATTCCCCATGAAACCTT-3’ and 3’- GGGACTTAATCAACGCAAGC-5’.

**Molecular Modeling**

Static protein/flexible ligand modeling of the interaction of GSNO with AKR1A1 was performed using Maestro 9.9 software. The AKR1A1 crystal structure (PDB: 3H4G) was prepared by removal of H2O and fidarestat from the PDB file. Original hydrogens were removed in Maestro and replaced, bond orders assigned, and the structure minimized. The docking grid was prepared around the active site at X = -2.099, Y = -24.407, Z = -6.508. GSH structure was obtained from PubChem and the GSNO structure was created from the GSH structure in Maestro. GSNO was prepared for docking in Maestro using the ligand preparation function. GSNO was docked to the active site grid using XP Glide Docking with post-docking minimization.
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The abbreviations used are: Ahd6: Yeast Alcohol dehydrogenase 6; AKR1A1: Aldo-keto reductase family 1 member A1; CBR1: Carbonyl reductase 1; CoA: Coenzyme A; GSH: Glutathione; GSNO: S-nitrosoglutathione; GSNOR: GSNO reductase; NADH/NAD+: Nicotinamide adenine dinucleotide; NADPH/NADP+: Nicotinamide adenine dinucleotide phosphate; NO: Nitric oxide; SCoR: SNO-CoA reductase; SNO: S-nitrosothiol; SNO-CoA: S-nitroso-coenzyme A; Trx: Thioredoxin; TrxR: Thioredoxin reductase
Table 1. Purification of NADPH-dependent GSNO reductase activity from bovine kidney

| Purification step                              | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Fold purification |
|-----------------------------------------------|-------------|--------------------|------------------------|------------------------------|-----------|-------------------|
| 1. Kidney soluble lysate                      | 105         | 5533.5             | 66.78                  | 0.012                        | 100       | 1                 |
| 2. Ammonium sulfate (30 to 60%) fractionation | 34.5        | 2070.3             | 49.68                  | 0.024                        | 74.44     | 2                 |
| 3. HiPrep Q fast flow                         | 22.5        | 91.8               | 41.35                  | 0.456                        | 61.94     | 37.68             |
| 4. HiPrep Phenyl fast flow                    | 23          | 15.7               | 17.7                   | 1.13                         | 26.52     | 93.38             |
| 5. Mono Q                                     | 2.8         | 3.53               | 15.06                  | 3.43                         | 17.87     | 283.6             |
| 6. HiTrap Phenyl HP                            | 2.75        | 0.536              | 4.746                  | 8.916                        | 7.1       | 736.8             |
| 7. G-200 Superdex                              | 1.8         | 0.24               | 2.94                   | 12.33                        | 4.39      | 1019              |
| Enzyme | Substrate | $K_m$ (µM)$^a$ | $k_{cat}$ (min$^{-1}$)$^a$ |
|--------|-----------|---------------|-----------------|
| WT     | GSNO      | 184 ± 8       | 948             |
|        | SNO-CoA$^b$ | 58 ± 4$^a$    | 959$^a$         |
| K127A  | GSNO      | 336 ± 24      | 953             |
|        | SNO-CoA$^b$ | 410 ± 27$^a$  | 750$^a$         |
| R312A  | GSNO      | 943 ± 104     | 383             |
|        | SNO-CoA$^b$ | 132 ± 11$^a$  | 410$^a$         |

$^a$ $K_m$ and $V_{max}$ were determined from Michaelis-Menten curves using GraphPad Prism 7. $k_{cat}$ was calculated by dividing $V_{max}$ by the enzyme concentration in each assay. Enzyme assays were performed in triplicate. $^b$ SNO-CoA kinetic data are taken from reference (14). See Table S2 for kinetic data from other mutant enzymes.
Figure 1. NADPH-dependent GSNO reductase activity in murine tissues. (A, B) Murine tissue extracts were incubated with 0.1mM NADPH (A) or NADH (B) in the absence or presence of 0.2 mM GSNO, and NADPH consumption (A) or NADH consumption (B) (absorbance at 340 nm) was followed over time. Auranofin (2 µM) (A) and GSNO R /- extracts (A, B) were used to rule out GSNO catabolizing activities by thioredoxin/thioredoxin reductase and GSNO R, respectively. Values are mean +/- SD for n=3. ** = p <0.01 by one-way ANOVA with Tukey’s correction for multiple comparisons. (B) In the heart, NADH-dependent GSNO reductase activity was not detected (n.d.) since NADH oxidase activity alone was greater than combined NADPH and GSNO consumption. Values are mean +/- SD for n=3. **** = p <0.0001, *** = p <0.001, ** = p <0.01 by T-test using Holm-Sidak method for determining significance. (C) GSNO-coupled protein S-nitrosylation is regulated by novel GSNO reductase activity. Representative Coomassie-stained SDS-PAGE gel of SNO-proteins enriched by SNO-RAC following incubation of GSNO R/ kidney lysates (0.5 mg/ml) for 10 min with 0.1 mM GSNO alone or in presence of 0.1 mM NADPH. The lysates were pre-incubated with 2 µM auranofin or vehicle (DMSO) for 30 minutes to inhibit thioredoxin/thioredoxin reductase-dependent SNO-protein denitrosylation. Results are representative of 3 independent experiments.
Figure 2. Purification of NADPH-dependent GSNO reductase activity. (A) Representative Coomassie-stained SDS-PAGE gel corresponding to the chromatographic purification scheme of NADPH-dependent GSNO reductase activity (described in Table 1), which yielded a highly purified species from a crude kidney extract (lane 1) that was identified as aldo-keto reductase 1A1 (AKR1A1) (lane 7) (see also Fig. S1, Table S1).
Novel GSNO reductase

A
Depleting Ab: IgG AKR1A1
AKR1A1 37 -
β-actin 50 -

B
GSNO Reductase Activity
(%) re. IgG control

C
+/+  +/-  -/-
AKR1A1 37 -

D
GSNO Reductase Activity
(% re. +/+)

E
NADPH:  -   -   -   -   +   +   +   -   -   -   -   +   +
GSNO:    -   +   +   +   +   +   -   -   +   +   +   -
AKR1A1:  +/-  +/-  +/-  +/-  +/-  +/-  +/-  +/-  +/-  +/-  +/-  +/-
Ascorbate: +  +  +  +  +  +  -  -  -  -  -  -

F
NADPH-dependent
GSNO Reductase Activity
(moles/min/mg)

G
AKR1A1
+/+  +/-  -/-

H
GSNOR++   GSNOR-/-

I
AKR1A1 protein expression
(Fold change re. +/+)

16
Figure 3. AKR1A1 is an NADPH-dependent GSNO reductase in mammals. (A) Representative western blot analysis following immunodepletion of AKR1A1 from wild-type mouse kidney extracts. (B) Relative NADPH-dependent GSNO reductase activity in IgG or AKR1A1 immunodepleted kidney extracts from panel A. Bars represent mean ± S.D for n=3. **** = p <0.0001 by Student’s t-test. (C) Representative western blot analysis of kidney extracts from AKR1A1+/+, AKR1A1+/-, and AKR1A1−/− mice. Example shown is representative of 3 independent experiments. (D) Relative NADPH-dependent GSNO reductase activity in kidney extracts from AKR1A1+/+, AKR1A1+/-, and AKR1A1−/− mice. Bars represent mean ± S.D for n=3. **** = p <0.0001 by one-way ANOVA with Tukey’s correction for multiple comparisons. (E) Representative Coomassie-stained SDS-PAGE gel illustrating SNO-proteins isolated by SNO-RAC following treatment of kidney extracts from AKR1A1+/+ and AKR1A1−/− mice with 0.1 mM GSNO (in the presence or absence of 0.1 mM NADPH). Results are representative of three independent experiments. (F) NADPH-dependent GSNO reductase activity across various tissues from AKR1A1+/+ and AKR1A1−/− mice. Extracts were incubated with 0.2 mM GSNO and 0.1 mM NADPH. Bars represent mean ± S.D for n=3. ** = p <0.01 and *** = p <0.001 by Student’s t-test. (G) Representative western blot analysis of various tissue extracts from AKR1A1+/+ and AKR1A1−/− mice. Example is representative of 3 independent experiments. (H) Western blot analysis for AKR1A1 expression in liver extracts from GSNOR+/+ or GSNOR−/− mice. (G) Quantification of AKR1A1 expression in liver extracts from GSNOR+/+ or GSNOR−/− mice (panel H). Bands (n=5) were quantified using ImageJ. **** = p <0.0001 by Student’s t-test.
Figure 4. Product and kinetic analysis of AKR1A1 GSNO reductase activity. (A) Glutathione sulfinamide was identified by MS as the major stable product of GSNO reduction by purified AKR1A1 (see Fig. S3 for product analysis). (B) NADPH consumption (as measured by change in absorbance at 340nm wavelength) by purified AKR1A1WT or AKR1A1Y50A (catalytically dead mutant) over 10 minutes in the presence of 0.1 mM GSNO and 0.1 mM NADPH. (C) Stoichiometry of NADPH:GSNO in AKR1A1-catalyzed GSNO reduction. Sequential additions of 0.084 mM NADPH to an excess of GSNO led to mean consumption of 0.073 ± 0.0018 mM of GSNO, demonstrating a stoichiometry near 1:1. Results shown are representative of two independent experiments. (D and E) Kinetic analysis of GSNO reductase activity by purified bovine AKR1A1 (D) and purified human AKR1A1 (E). Enzyme assays were performed in duplicate (D) or triplicate (E). Kinetic values were calculated using GraphPad Prism 7.
Figure 5. Molecular modeling and mutagenic analysis of AKR1A1 GSNO reductase activity. (A) Binding model of GSNO in the AKR1A1 active site. Putative interacting residues and the SNO-CoA binding residue (K127) are highlighted in red. NADPH carbons are colored green. (B) Catalytic efficiency ($K_{cat}/K_m$) of AKR1A1 mutants expressed relative to AKR1A1$^{WT}$. $K_{cat}$ and $K_m$ were determined from two independent purifications (calculated using GraphPad Prism 7) and used to generate an average catalytic efficiency for each enzyme. (C) Kinetic analysis of GSNO reductase activity by AKR1A1$^{WT}$ and AKR1A1$^{K127A}$. Enzyme assays were performed in triplicate. AKR1A1$^{WT}$ curve is re-plotted from Fig. 4E for comparison. Kinetic values are listed in Table 2. (D) Kinetic analysis of GSNO reductase activity by AKR1A1$^{WT}$ and AKR1A1$^{R312A}$. Enzyme assays were performed in triplicate. AKR1A1$^{WT}$ curve is re-plotted from Fig. 4E for comparison. Kinetic values are listed in Table 2. (E) Relative GSNO reductase activity in the presence of increasing concentrations of glutathione (GSH) or coenzyme A (CoASH). Increasing amounts of GSH and CoASH were added to a reaction mix of 100 µM GSNO/NADPH and 20 nM AKR1A1$^{WT}$. Assays were performed in duplicate. IC50s were calculated in GraphPad Prism 7.
AKR1A1 is a novel mammalian S-nitroso-glutathione reductase
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