The role of S-nitrosoglutathione reductase (GSNOR) in human disease and therapy

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ABSTRACT
S-nitrosoglutathione reductase (GSNOR), or ADH5, is an enzyme in the alcohol dehydrogenase (ADH) family. It is unique when compared to other ADH enzymes in that primary short-chain alcohols are not its principle substrate. GSNOR metabolizes S-nitrosoglutathione (GSNO), S-hydroxymethylglutathione (HMGSH) (Hedberg et al., 2000), and a handful of alcohols (Adinolfi et al., 1984; Jensen et al., 1998). If we look beyond the direct actions of the enzyme itself, it quickly becomes apparent that GSNOR influences several downstream and parallel pathways (Figure 1). One of the most important is GSNOR’s regulation of GSNO, and by extension, nitric oxide (NO) and protein S-nitrosation. NO is a reactive nitrogen species (RNS) that is critical to the normal function of most cell types (Beckman & Koppenol, 1996; Moncada et al., 1991; Radi et al., 1991; Salvador Moncada, 1994). It is a powerful smooth muscle relaxing agent (Bradley et al., 1998; Buxton et al., 2001; Ricciardolo et al., 2004; Tomita et al., 2002), cardiopulmonary regulator (Liu et al., 2004; Tamargo et al., 2010), neuroeffector (Bredt & Snyder, 1992; Corti et al., 2014) and immune system modulator (MacMicking et al., 1997). NO is likely carried as GSNO from endothelium, and other sources, and acts as a stable NO reserve (Broniowska et al., 2013; Smith & Marletta, 2012). GSNO can transfer its NO moiety to a cysteine thiol, resulting in the posttranslational modification (PTM) S-nitrosation/S-nitrosylation (Stamler et al., 1992). S-nitrosation describes a thiol (e.g. cysteine) converted to a S-nitrosothiol (RSNO) by a one-electron oxidation from the NO radical (Smith & Marletta, 2012). The term nitrosylation describes addition of an NO group to a metal centered protein such as guanylyl cyclase (Martinez-Ruiz & Lamas, 2004). Researchers have used both terms to describe NO addition to a protein thiol. We employ S-nitrosation to refer to protein modifications on cysteine residues. Protein S-nitrosations are also referred to in the literature in a fashion that takes into account protein and non-protein nitrosations (e.g. RSNO). We employ the term RSNO as it appears in the literature.

Introduction
S-nitrosoglutathione reductase (GSNOR) is an important regulator of human health and disease. The modulation of protein S-nitrosation by GSNOR contributes to a host of maladies and can be exacerbated by the dysregulation of GSNOR. In recent years, much effort has been dedicated to identifying a safe and efficacious means to alter GSNOR activity. A myopic investigation of GSNOR would reveal little more than its inherit ability to metabolize S-nitrosoglutathione (GSNO) (Jensen et al., 1998), S-hydroxymethylglutathione (HMGSH) (Hedberg et al., 2000), and a handful of alcohols (Adinolfi et al., 1984; Jensen et al., 1998). If we look beyond the direct actions of the enzyme itself, it quickly becomes apparent that GSNOR influences several downstream and parallel pathways (Figure 1). One of the most important is GSNOR’s regulation of GSNO, and by extension, nitric oxide (NO) and protein S-nitrosation. NO is a reactive nitrogen species (RNS) that is critical to the normal function of most cell types (Beckman & Koppenol, 1996; Moncada et al., 1991; Radi et al., 1991; Salvador Moncada, 1994). It is a powerful smooth muscle relaxing agent (Bradley et al., 1998; Buxton et al., 2001; Ricciardolo et al., 2004; Tomita et al., 2002), cardiopulmonary regulator (Liu et al., 2004; Tamargo et al., 2010), neuroeffector (Bredt & Snyder, 1992; Corti et al., 2014) and immune system modulator (MacMicking et al., 1997). NO is likely carried as GSNO from endothelium, and other sources, and acts as a stable NO reserve (Broniowska et al., 2013; Smith & Marletta, 2012). GSNO can transfer its NO moiety to a cysteine thiol, resulting in the posttranslational modification (PTM) S-nitrosation/S-nitrosylation (Stamler et al., 1992). S-nitrosation describes a thiol (e.g. cysteine) converted to a S-nitrosothiol (RSNO) by a one-electron oxidation from the NO radical (Smith & Marletta, 2012). The term nitrosylation describes addition of an NO group to a metal centered protein such as guanylyl cyclase (Martinez-Ruiz & Lamas, 2004). Researchers have used both terms to describe NO addition to a protein thiol. We employ S-nitrosation to refer to protein modifications on cysteine residues. Protein S-nitrosations are also referred to in the literature in a fashion that takes into account protein and non-protein nitrosations (e.g. RSNO). We employ the term RSNO as it appears in the literature.

Alcohol dehydrogenase family overview
The alcohol dehydrogenase (ADH) family of enzymes have been investigated for well over a century...
They are evolutionarily conserved from bacteria to man (González-Duarte & Albalat, 2005; Liu et al., 2001) and are categorized into five distinct classes that contain seven known isoforms (Table 1). ADH enzymes perform several important functions in human cells. The most well studied of these is the metabolism of short chain alcohols. Ethanol, being of significant cultural relevance due to its widespread consumption and abuse (Oscar-Berman & Marinkovic, 2003), has led to an extensive investigation of the entire ADH family. Most ADH enzymes have some affinity for ethanol. In hepatocytes, ADH1A (formerly ADH1), ADH1B (formerly ADH2) and ADH1C (formerly ADH3), are responsible for the oxidative catabolism of ethanol to acetaldehyde before further processing in the Krebs cycle, or elimination (Cederbaum, 2013). ADH4, a class II ADH (Svensson et al., 2001) whose sequence is 70% homologous to ADH1, catalyzes the oxidation of retinol (Vitamin A), and bolsters ethanol metabolism in the liver (Ramchandani et al., 2001). Numerous single nucleotide polymorphisms (SNPs) in the genes encoding the ADH family affect the rate of ethanol metabolism. These SNPs have been linked to some forms of alcoholism and cancer (Edenberg, 2007; Hurley & Edenberg, 2012). Other ADH SNPs have been correlated with schizophrenia, Parkinson’s disease, asthma and autism in certain populations (Bowers et al., 2011; Buervenich et al., 2000; Wu et al., 2007; Zuo et al., 2013). GSNOR (ADH5), the focus of this review, is differentiated from other ADH enzymes in that primary short chain alcohols, in particular ethanol, are not its principal substrate. ADH6 has been identified in both fetal and adult livers, but its function remains unclear as this enzyme has yet to be isolated for biochemical analysis (Edenberg, 2007; Östberg et al., 2016). A recent examination of ADH6 has provided evidence that it may act as an S-nitroso-CoA reductase (Anand et al., 2014). Similarly, ADH7’s function remains elusive. Available data suggest ADH7 may serve a role in seemingly disparate cellular functions and diseases, such as: first pass gastric metabolism of ethanol (Lee et al., 2006), retinol metabolism (Chase et al., 2009), Parkinson’s disease (Buervenich et al., 2000) and even personality traits in some individuals with substance dependence (Luo et al., 2008). Clearly, the ADH family of enzymes performs a diverse and important role in

Figure 1. GSNOR in the cell. One of the principle functions of GSNOR is to metabolize GSNO. SNPs in ADH5 can affect expression of GSNOR in the cell, which in turn alters the concentration of GSNO and total levels of RSNOs (term to include nitrosation of cysteine residues, e.g. SNO). Inhibitors of GSNOR increase available GSNO and increase total RSNOs (see colour version of this figure at www.tandfonline.com/lbm).
the cellular metabolism of endogenous and exogenous chemicals. Here we focus on the function, significance and therapeutic potential of modulating GSNOR activity.

**Nomenclature of alcohol dehydrogenases**

The ADH family of enzymes has had several overlapping naming schemes in the past (Holmquist & Vallee, 1991; Staab et al., 2008). This has led to ambiguity in the literature and is due in part to the fact that naming assessments have historically been guided by substrate specificity, phylogenic classification and publication date. GSNOR was not disambiguated from glutathione-dependent formaldehyde dehydrogenase (FDH) until 1989 when it was found that these two proteins were in fact the same enzyme (Koivusalo et al., 1989). A formal attempt to reconcile the nomenclature began in 1999 when it was proposed that ADH proteins use numeric Arabic designators to identify each class of enzyme (Duester et al., 1999). In recent years, the research community has generally adopted the gene naming guidelines put forth by the Human Genome Organization’s Gene Nomenclature Committee (Wain et al., 2002). Of all the ADH enzymes, GSNOR naming is particularly convoluted in this respect. While this protein is still sometimes referred to in the literature by its nonstandard name, ADH3 (as in class III ADH), the official gene designator is now ADH5, and the protein is GSNOR, ADH5 or alcohol dehydrogenase 5 (class III) \( \chi \)-polypeptide. It can also be found in the literature under several other monikers: formaldehyde dehydrogenase (FDH or FALDH); alcohol dehydrogenase X (ADHX); alcohol dehydrogenase class-3 (ADH-3); \( \chi \chi \chi \)-ADH (homodimeric \( \chi \)-ADH); ADH 5; glutathione-dependent formaldehyde dehydrogenase (GSH-FDH); and S-(hydroxymethyl) glutathione dehydrogenase (EC 1.1.1.284). For purposes of clarity, this review will address the gene as ADH5, and the protein as ADH5 or GSNOR.

**ADH5: structure/localization**

ADH5, the gene that encodes GSNOR, is located on the reverse strand of chromosome 4 (4q23 – chr4:99993567 – 10000985) (Smith, 1986). ADH5 is tandemly aligned in the same orientation as the other genes that encode for the entire family of ADH enzymes. Phylogenetic analysis of the ADH5 locus revealed that GSNOR evolved independently from class I and II ADH (Adinolfi et al., 1984), and it is highly conserved across most vertebrate species (Foglio & Duester, 1996). GSNOR has a molecular weight of 39,724 Da and is translated to a 374 amino acid enzyme (UniProtKB identifier: P11766) via 9 exons (Hur & Edenberg, 1992). Glu-67 and Arg-368 are highly conserved essential amino acids important to the catalytic mechanism of this enzyme (Sanghani et al., 2006). Splice variants of ADH5 exist and result in the production of truncated proteins; however, their functional relevance has not been documented (Höög et al., 2001).

GSNOR functions as a homodimer (Figure 2) (Yang et al., 1997) and is localized to the nucleus and cytoplasm (Fernández et al., 2003). Amino acid substitutions in the subunit interacting portions of the coenzyme-binding domain prevent heterodimeric variants from being generated with other ADH enzymes (Julia et al., 1988). Each subunit binds a catalytic and structural \( \text{Zn}^{2+} \) cofactor (Kaiser et al., 1988; Östberg et al., 2016), for a total of four \( \text{Zn}^{2+} \) ions per functional enzyme. In addition to \( \text{Zn}^{2+} \), GSNOR also requires a coenzyme that can vary based upon the substrate. These include: nicotinamide adenine dinucleotide (NAD+), its reduced form NADH, NADPH + H+ or NAD(P)+ (Gupta & Igamberdiev, 2015; Hedberg et al., 2003; Jensen et al., 1998; Sanghani et al., 2000).
In general, ADH enzymes are highly expressed in the liver, the upper digestive tract and the kidneys (Zuo et al., 2013). ADH5 RNA has been recognized in all major human tissue types with protein expression highest in smooth muscle, liver, epididymis, kidney and testis (Giri et al., 1989). GSNOR is an important negative regulator of neuronal differentiation during development (Wu et al., 2014) and is the only known ADH enzyme present in the brain (Beisswenger et al., 1985; Galter et al., 2003). Conversely, GSNOR protein expression is negligible or non-existent in skeletal muscle, lymph nodes, spleen, bone marrow, cerebellum and the lateral ventricle (If & Wb, 2017).

Substrates

S-nitroso glutathione/formaldehyde

As with most enzymes, GSNOR has a varying degree of affinity for several substrates. The two primary targets of GSNOR are GSNO, and HMGSH, the spontaneous adduct of formaldehyde and glutathione. HMGSH binds at the zinc active site and interacts with the highly conserved residues Arg114/115, Asp55, Glu57 and Thr46 (Engeland et al., 1993; Sanghani et al., 2002). That being said, the rate of substrate conversion ($K_{cat}$) is about 20-fold higher for GSNO over HMGSH (Green et al., 2012; Hedberg et al., 2003; Salisbury & Bronas, 2015; Sanghani et al., 2000; Staab et al., 2008). Both reactions are dependent on an abundant source glutathione (GSH) in the cell. GSH is the major thiol in mammalian cells and while concentrations can reach as high as 10 mM (Bateman et al., 2008), they are typically 1 mM. Under stress conditions, the concentration can fluctuate dramatically and drive GSNO toward atypical reactions (Figure 3) (Salisbury & Bronas, 2015; Staab et al., 2009). The enzymatic activity of human recombinant GSNOR for GSNO exhibits a $K_m$ of approximately 27 μM and a $k_{cat}$ value of between 2400 and 12,000 min$^{-1}$ (Fernández et al., 2003; Hedberg et al., 2003).

Alcohols

GSNOR more readily acts upon alcohols of greater chain length than class I ADH enzymes (Figure 4). This is due in part to a longer span between the binding and active site of the enzyme (Salisbury & Bronas, 2015), as well as amino acid substitutions that affect binding affinity (Julià et al., 1988; Östberg et al., 2016). As a result of these evolutionary divergences, GSNOR is not optimized for metabolizing short-chain alcohols. Consequently, it is not a misnomer to identify GSNOR as an ADH. GSNOR metabolizes both ethanol and medium/long chain alcohols (preferring a double-bond in the beta position). The active site of GSNOR cannot be saturated by ethanol (Beisswenger et al., 1985), and the high activity of class I ADH enzymes toward ethanol minimizes the functional role of ethanol metabolism by GSNOR. Several $K_m$ values for EtOH (all $>$2 M) (Lee et al., 2003; Sharma et al., 1989) have been reported in the literature, with a $k_{cat}$ of 33 ± 3 min$^{-1}$ (Beisswenger et al., 1985; Lee et al., 2003). GSNOR’s ability to metabolize EtOH is far surpassed by those of class I ADH enzymes whose $K_m$ values range from 0.05 to 40 mM. As such, medium and long chain alcohols (4 carbons) (Holmquist & Vallee, 1991; Salisbury & Bronas, 2015; Theorell et al., 1969; Wagner et al., 1984) are more freely oxidized by GSNOR (Staab et al., 2009).

Other substrates

As with most enzymes, the entire cohort of ADH5 substrates is not fully known. Additional classes of molecules such as $\omega$-hydroxy fatty acids (Achkor et al., 2003; Boleda et al., 1993; Moullis et al., 1991) exhibit a limited affinity for the enzyme. The ability of GSNOR to metabolize retinol remains in question. ADH7 (a class IV ADH) is the primary ADH accountable for retinol metabolism (Cederbaum, 2013), but there is evidence to support GSNOR’s contribution in the retinoid-signaling pathway. Studies have shown that ADH5$^{-/-}$ null mice exhibit reduced retinoic acid production (Molotkov et al., 2002), and the presence of ADH5 transcript in human fetal lungs correlates with a decrease in the presence of retinol (Coste & Labbe, 2011). Ultimately, the exact nature of relationship between GSNOR and retinol is still under investigation (Boleda et al., 1993; Canestro et al., 2010; González-Duarte & Albalat, 2005).
GSNOR: health and disease

GSNOR is integral to the modulation of \( \cdot \)NO in the cell. \( \cdot \)NO is produced enzymatically in many cell types (Schmidt & Walter, 1994). Free \( \cdot \)NO is a highly reactive uncharged radical with a half-life of \(~1\text{–}5\) second \textit{in vivo} (Kelm & Schrader, 1990), and will often establish a stable RSNO equilibrium with GSH in the form of GSNO (Wink & Mitchell, 1998).
plays a critical role in smooth muscle relaxation (Bradley et al., 1998; Buxton, 2004; Liu et al., 2016; Ricciardolo et al., 2004; Tomita et al., 2002) cardiopulmonary regulation (Rastaldo et al., 2007; Sears et al., 2004; Tamargo et al., 2010), neuronal signaling (Shahani & Sawa, 2011), as well as dozens of other intra/extracellular functions (Pacher et al., 1995; Salvador Moncada, 1994). The dysregulation of NO production and metabolism can lead to drastic changes in protein S-nitrosation (Foster et al., 2003, 2009), an important PTM, and can have numerous other downstream consequences.

**Oxidative/nitrosative stress**

The dysregulation of GSNO through aberrant GSNOR modulation, when combined with oxidative stress, can further exacerbate disease. During conditions of cellular stress RNS, such as peroxynitrite (ONOO–), are formed when NO reacts with superoxide (O2•–) (Squadrito & Pryor, 1998). Not only does oxidative stress commandeer available NO and GSH (Rahman & MacNee, 2000), but peroxynitrite can cross the cell membrane and react directly with protein thiols (Alvarez & Radi, 2003), which may prevent S-nitrosation. RNS also induce S-glutathionylation of protein thiols (Dalle-Donne et al., 2009), further depleting the GSH pool (Klatt & Lamas, 2000). Decades of research have left little question as to detrimental effects of oxidative/nitrosative stress (Dalle-Donne et al., 2006; Guzik et al., 2002; Münzel et al., 1997), and the mechanistic underpinnings of this process have been thoroughly investigated (Apel & Hirt, 2004; Valko et al., 2007). For the purpose of this review, it should be noted that this process can alter the levels of NO and GSH in the cell, which in turn can affect NO/GSNO signaling.

**GSNO and S-nitrosation**

Any investigation into the modulation/activity of GSNOR would not be complete without mention of S-nitrosation. The study of this PTM and its influence on normal cell-signaling and disease has significantly impacted research and medicine for over 25 years (Broniowska & Hogg, 2012; Foster et al., 2009; Stamler et al., 1992).

The detection and quantitation of RSNOs in biological systems are inherently challenging. The biotin switch technique (Jaffrey & Snyder, 2001), in which S-nitrosated cysteines are reduced and biotinylated, provides a simple and elegant method for the qualitative detection of S-nitrosated proteins. An analysis of a wide variety of RSNO measurement techniques, including the biotin switch, has established that artifacts are common when measuring RSNOs and it is not always possible to identify which thiols have been S-nitrosated (Giustarini et al., 2003). Newer techniques have become available in recent years (Chen et al., 2013; Devarie-Baez et al., 2013), such as tandem mass spectrometry (MS/MS) of S-nitrosated protein thiols (Murray et al., 2012; Ulrich et al., 2013), that are highly quantitative. Beyond the problem of quantitation, it has been proposed that other thiol modifications such as dithiol/disulfide exchange, S-glutathionylation and oxidation, may affect signaling more readily than do RSNOs (Lancaster, 2008), and should be investigated along with S-nitrosation.

As with phosphorylation, S-nitrosation regulates cellular mechanisms and affects protein–protein interactions. The intracellular availability of NO and its functional derivatives, like GSNO, affect protein S-nitrosation (Broniowska & Hogg, 2012; Hess et al., 2005; Thomas & Jourd’heuil, 2012). GSNOR is a potent negative regulator of GSNO in smooth muscle (Que et al., 2009). The aberrant expression of ADH5, as with many ADH subclasses, is associated with disease (Jelski & Szmitkowski, 2008; Jelski et al., 2009; Laniewska-Dunaj et al., 2013). In fact, the deletion of the ADH5 gene increases both the levels of GSNO and total protein S-nitrosation in vivo (Liu et al., 2001). Protein S-nitrosation is of intense interest to researchers and clinicians as the hypo/hyper-S-nitrosation of a diverse set of proteins, spanning nearly every tissue type, can have a drastic effects in disease (Foster et al., 2009). Some of these include: Type 2 diabetes (Carvalho-Filho et al., 2005), sickle cell anemia (Bonaventura et al., 1999, 2002), ventricular arrhythmia in individuals with the Duchenne muscular dystrophy (Faucconnier et al., 2010), cell death and survival pathways (Iyera et al., 2011), post-infarct cardio-protection (Methner et al., 2014), pregnancy/parturition (Ulrich et al., 2013) and many others. Interestingly, GSNOR itself is a cysteine rich protein that is S-nitrosated by GSNO, which in turn initiates a feedback loop that affects GSNOR expression (Guerra et al., 2016) and activity (Brown-Steinke et al., 2010). Although it is beyond the scope of this review, it should be noted that GSNOR dysregulation in plants can result in significant biotic and abiotic nitrosative events that affect growth, development and survival (Leterrier et al., 2011; Shi et al., 2015; Yun et al., 2016).

**GSNOR dysregulation**

GSNOR dysregulation has been implicated in numerous disease states (Figure 5). The use of models and ADH5−/− knockout animals has uncovered surprising and valuable data related to GSNOR function. RSNO levels, as
well as canonical NO-mediated pathways, are severely altered when GSNOR activity is modulated.

**Cardiovascular health**

One of the major organs affected by GSNOR is the heart and surrounding vascularity. It has long been known that -NO and S-nitrosation protect the body from cardiovascular disease. Following myocardial infarction, ADH5−/− mice exhibit enhanced cardiac regenerative capabilities as a result of increased cardiac stem cell turnover (Hatzistergos et al., 2015), as well as a reduction in myocardial infarct size and higher coronary vascular density (Lima et al., 2009). Moreover, de-S-nitrosation of cardiac ryanodine receptor 2 (RyR2) in ADH5−/− mice results in decreased peripheral vascular tone due to calcium “leak” (Beigi et al., 2012). In skeletal muscle only about 1 in 50 cysteines on the ryanodine receptor are S-nitrosated, indicating that this PTM, even when conservatively distributed, can drastically alter protein function (Sun et al., 2001). Taken together this data suggests that RyR2 S-nitrosation modulates calcium storage in the sarcoplasmic reticulum. There is clearly a complex relationship between the correlative observation of an increase in S-nitrosation and GSNOR dysregulation.

**Immune system**

GSNOR performs an important protective role in the immune system’s development of lymphocytes. ADH5−/− KO mice show increased RSNO production that decreases CD4 single-positive thymocyte development, and increases lymphocytic apoptosis (Yang et al., 2010). Damage to immune cells from nitrosative stress in ADH5−/− mice results in a significant increase in the animal’s susceptibility to pulmonary infection by *K. pneumoniae* as well as multi-fold increases of the bacteria in the spleen and blood, resulting in increased inflammation (Tang et al., 2013a, 2013b). Enhanced nitric oxide synthase (NOS) 2 activity in monocytes and macrophages increases -NO production and elicits a cytostatic or cytotoxic response against bacteria, viruses and other intruders, but also increases inflammation (MacMicking et al., 1997). The bronchoalveolar lavage fluid of asthmatics consists of high macrophage levels as well as significantly increases GSNOR activity (Que et al., 2009). Inhibiting GSNOR in these patients increases total RSNOs and restores inflammatory markers to near baseline levels while limiting ova-induced NFκB activation (Blonder et al., 2014). Ultimately, the balance between GSNOR activation and inhibition is critical in maintaining balance in the immune system.
**Brain development and function**

GSNOR regulation in the brain affects a broad swath of cellular functions ranging from neural development and maturation to other neurodegenerative diseases more typically associated with adult and geriatric populations. These disease states are often the result of aberrant protein S-nitrosation caused by the dysregulation of GSNOR. For instance, in developing and adult mouse brains the overexpression of GSNOR results in decreased neuronal differentiation in part due to de-S-nitrosation of histone deacetylase 2 (HDAC2) (Wu et al., 2014). Conversely, ADH5−/− mice exhibit neuromuscular atrophy as a result of a decrease in muscle mass, while also presenting with neuropathic behavior (Montagna et al., 2014). In Drosophila, GSNOR overexpression results in visual pattern memory defects which can be rescued by co-expression of cyclic-GMP dependent protein kinase G (PKG) (Hou et al., 2011). This occurs independently from neuronal development and implies an adjacent regulatory role for GSNOR in the PKG phosphorylation pathway. Neuronal homeostasis is also affected by GSNOR. In a Parkinson’s disease model using neuronal (SH-SY5Y) cells, a decrease in GSNOR availability results in activation of nuclear factor Nrf2 ([erythroid-derived 2]-like 2), which regulates the expression of antioxidant proteins (Rizza et al., 2015). Interestingly, GSNOR may also affect the phosphorylated state of platelet-derived growth factor receptor-β (Palmer et al., 2015) in the brainstems of mice during hypoxic exposure. When these data are considered as a whole, it is apparent that deviating GSNOR activity and expression from baseline can have drastic consequences in both the developing and mature brain.

**Cancer**

The link between GSNOR dysregulation and cancer is not well understood. GSNOR deficiency has been known to affect the rate of genomic mutations in mice by increasing the frequency of A:T to T:A transposition (Leung et al., 2013). This may be the result of a GSNOR-mediated reduction in activity of the DNA repair protein O6-alkylguanine-DNA alkyl transferase which can lead to an increase in the rate of human hepatocellular carcinoma (HCC) (Tang et al., 2012; Wei et al., 2010, 2011). Pharmacologic inhibition of inducible NOS (iNOS) when GSNOR is down-regulated shows strong potential as a therapeutic for those patients with HCC (Tang et al., 2013a). As with HCC, some types of breast cancer are linked to a decrease in GSNOR expression. Specifically, high levels of human epidermal growth factor receptor 2 (HER2) expression in breast tumors is associated with low GSNOR expression and an increase in apoptosis-related protein S-nitrosation (Cañas et al., 2016). This study also determined that an increase in GSNOR expression in HER2 tumors correlates with higher patient survival and begs the question as to whether or not NOS inhibition would also serve this population well. These examples are of course complicated by the fact that NO is a pleiotropic regulator of gene function and the modulation of GSNOR by GSNOR can have both cytostatic and cytotoxic effects on tumor survival (Xu et al., 2002). To this point, GSNOR is effective at removing formaldehyde, a known carcinogen, from the cell; however, ADH5 polymorphisms do not significantly affect an individual’s capacity to protect against DNA damage when exposed to formaldehyde (Xie et al., 2010). Furthermore, ADH5−/− mice are known to generate DNA damage when formaldehyde forms and adduct with guanine to create N2-hydroxymethyl-dG which can result dysfunction of hepatocytes and nephrons (Pontel et al., 2015).

**Asthma and single nucleotide polymorphisms**

SNPs can alter the transcriptional output of a gene as well as the structure/function of proteins they encode. Several SNPs in the promoter and 3’ UTR of the ADH5 gene can result in the aberrant expression of GSNOR (Choudhry et al., 2010). Of particular interest is the observation that airway hyperresponsivity in wild-type mice correlates with increased expression of GSNOR and decreased RSNO production, while ADH5−/− mice are protected from airway hyperresponsiveness and maintain higher total RSNO levels (Que et al., 2005). In humans, GSNOR upregulation can lead to changes in airway smooth muscle tone in asthmatics (Henderson & Gaston, 2005; Wu et al., 2007). A study involving Mexican children with asthma who possess SNPs in the promoter region of ADH5 at suspected NF-xB binding sites (rs2602899 and rs2873101), were found to exhibit a decreased relative risk of asthma due to suppressed GSNOR production (Wu et al., 2007). Interestingly, alternative SNPs (rs1154404 and rs28730619) were associated with an increase in childhood asthma risk, although the mechanism behind this correlation has not been determined (Wu et al., 2007). Another study in African American children found that SNPs in ADH5 and the β2 adrenergic receptor gene are associated with acute response to asthma-specific therapy (Moore et al., 2009).

Looking beyond GSNO-mediated relaxation of airway smooth muscle we may also consider GSNOR’s ability to...
metabolize formaldehyde, a chemical known to induce bronchoconstriction after long term exposure at low concentrations (Leikau, 1992). It has been suggested that the presence of formaldehyde in airway smooth muscle may stoichiometrically favor bound NADH/GSNOR, thereby increasing GSNOR metabolism of GSNO, and by extension, promote smooth muscle contraction (Thompson & Grafström, 2007).

Regardless of the mechanism driving GSNOR-mediated consumption of GSNO in airway smooth muscle, it is easy to see why the inhibition of GSNOR has been of particular interest to researchers for its therapeutic potential as a smooth muscle relaxant.

Myoendothelial junctions

GSNOR plays an interesting role at myoendothelial junctions (MEJ) where it co-localizes with the hemichannel Connexin-43 (Cx43). Cx43 hemichannels form gap junctions between cells by linking to hemichannels in opposing membranes to couple endothelial and vascular smooth muscle cells and when Cx43 is S-nitrosated, this pore allows for the free movement of inositol triphosphate from vascular smooth muscle to endothelial cells. Due to the co-localization of GSNOR and Cx43 at the MEJ, basal NO availability at this site is blunted, which in turn increases the likelihood that Cx43 will not be S-nitrosated (Straub et al., 2011). This decreases channel permeability until Ca²⁺ levels increase as a result of smooth muscle cell stimulation, which in turn activates eNOS and increases the probability of Cx43 S-nitrosation.

Myometrium

NO is an important mediator of relaxation in the myometrium. It has been well established that NO relaxes vascular and gastrointestinal smooth muscle by activating soluble guanylyl cyclase (sGC), which in turn converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP), activating PKG, which in turn dephosphorylates the regulatory light chain of myosin via the amplified phosphatase activity of MYPT1 (pS695) (Grassie et al., 2011; Nakamura et al., 2007; Puetz et al., 2009; Roux et al., 2012). This is not the dominant NO-mediated relaxation pathway in uterine smooth muscle, however, NO can relax the myometrium even when sGC has been inhibited (Buxton et al., 2010). The pathway through which NO relaxes the myometrium independent of cGMP is unknown, but it is likely that the S-nitrosation of contractile proteins plays a role. It has been determined that the state of labor (full term versus preterm) can vastly alter the S-nitrosated protein landscape in uterine smooth muscle after exposure to GSNO (Ulrich et al., 2012). It is also well known that S-nitrosation can vary significantly based upon the cytoplasmic availability GSNOR (Broniewska & Hogg, 2012; Hess et al., 2005; Thomas & Jourd’heuil, 2012). Regardless of the pathway through which NO acts to relax uterine smooth muscle, it does beg the question as to whether or not inhibiting GSNOR, and thereby increasing intracellular availability of GSNO, may serve as an effective tocolytic strategy by promoting uterine quiescence through NO-mediated relaxation pathways. This notion is supported by data showing an increased expression of GSNOR in patients delivering spontaneously preterm.

Therapeutic inhibition of GSNOR

GSNOR is an attractive therapeutic target. GSNOR inhibition increases GSNO availability in the cell and in turn facilitates NO-mediated signaling pathways. Dozens of small molecules have been identified that can inhibit GSNOR to varying degrees (Green et al., 2012; Jiang et al., 2016; Sanghani et al., 2009; Sun et al., 2011a, 2011b, 2012). Two of these, N6022 (3-(5-(4-(1H-imidazo[4,5-c]pyridin-9(10H)-yl)phenyl)-1-(4-carbamoyl-2-methylphenyl)-1H-pyrrolo[2,3-y]propionic acid) and N91115 from Nivalis Pharmaceuticals show promise as potentially safe and effective GSNOR inhibitors that have undergone clinical trial for both the treatment of mild asthma (clinicaltrials.gov – NCT01316315), and cystic fibrosis in individuals who are heterozygous for the cystic fibrosis transmembrane conductance regulator (CFTR) gating mutation CFTRAF508+ (clinicaltrials.gov – N6022: NCT01746784; N91115: NCT02724527). Endogenous GSNO levels are low in the airways of cystic fibrosis patients (Grasemann et al., 1999) and GSNOR inhibition is an appealing alternative to the direct administration of GSNO (Snyder et al., 2002; Zaman et al., 2001, 2013). N6022 is well tolerated with minimal side effects, even at high concentrations, in both animals (Blonder et al., 2014; Colagiovanni et al., 2012) and humans (clinicaltrials.gov – NCT01147406, NCT01746784). Another GSNOR inhibitor, SPL-334 (4-[[2-[[2-(cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl][methyl]benzoic acid] from SAJE Pharmaceuticals (Baltimore, MD), is being tested as a therapeutic to treat allergic asthma and interstitial lung disease (ILD). Using an allergic asthma mouse model, intranasally administered SPL-334 decreased CD4+ Th2 cytokines, eosinophils, and mitigated the lung inflammatory response (Ferrini et al., 2013). Likewise, in a mouse model of ILD, SPL-334 functions as both a prophylactic agent and a therapeutic to
attenuate profibrotic cytokines and collagen accumulation in the lungs (Luzina et al., 2015). Unlike N6022 and N91115, SPL-334 is not in human clinical trials.

FDA-approved drugs are also being tested as potential GSNOR inhibitors. Nebivolol, a β1-adrenergic receptor antagonist used for the management of hypertension, has been shown to increase total RSNO levels in animal and cell models (Jiang et al., 2016). Our own investigation of GSNOR fails to confirm Nebivolol as an inhibitor of GSNOR in an enzyme activity assay. Since there are no FDA-approved GSNOR inhibitors, the repurposing of existing therapeutic agents that inhibit GSNOR and/or modulate GSNO and RSNOs is of interest.

When considering GSNOR inhibitors as therapeutic agents, it should be taken into consideration that enzymes other than GSNOR modulate -NO availability in the cell. -NO is critical to the normal function of most cell types, and as is often the case, there are multiple concurrent and complementary mechanisms to regulate -NO and RSNOs (Benhar et al., 2009; Liu et al., 2001). Two of the most well-known are thioredoxin-1 (Sengupta & Holmgren, 2012a, 2012b) and carbonyl reductase (Bateman et al., 2008). NOS, the predominate source of -NO in the body, can also be dysregulated in certain disease states, as can its substrate, l-arginine (Ckless et al., 2007). For instance, after stimulation of the cavernous nerve in ADHS+/− mice, eNOS phosphorylation did not increase as predicted (Musicki et al., 2016). Modulating GSNOR activity may insufficiently control, or even aggravate some conditions if these alternate -NO-regulators are the source of the disorder. Unfortunately, direct application of endogenous -NO-donors, such as GSNO, Cys-NO or SNO-albumin, as well as some exogenous donors, are of limited clinical value because they either degrade rapidly, cause intolerable side effects, or lead to a toxic systemic build up nitrates (Al-Sa‘doni, 2005).

The therapeutic inhibition of GSNOR to treat -NO-mediated disorders should be weighed carefully against potential contraindications. For example, the inhibition of GSNOR may increase a patient’s susceptibility to bacterial or viral infection. The inhibition of GSNOR will also increase total RSNO levels and this can have adverse effects in the body, especially if the drug is administered systemically and not targeted to a specific tissue type through means such as liposomal delivery. GSNOR regulation varies widely in different cancer types (Cañas et al., 2016; Tang et al., 2013a). Inhibiting GSNOR may lead to a further increase in GSNO at the tumor site which can favor angiogenesis (Prudente et al., 2017). Conversely, with disorders such as asthma and hypertension, GSNOR inhibition results in the desired relaxation of the smooth muscle.

Conclusions

-NO, and by extension -NO-donors, have been investigated intensely for over a century as therapeutics (Schmidt & Walter, 1994). -NO modulation not only affects traditional pathways connected to this highly reactive molecule, but it also drastically alters S-nitrosation levels in the cell. GSNOR is unique among the ADH family of enzymes in that it targets GSNO and varies the body’s response to endogenously generated -NO carried as GSNO. ADH5−/− animal and cell models have provided a unique window into the importance of GSNOR in nearly every tissue type. The up/down regulation of GSNOR in humans has also provided invaluable data to the medical and research communities concerning its role in disease states. There are currently no FDA-approved modulators of GSNOR; however, several drugs are being investigated, and some are in clinical trial. Indeed, our understanding of the dysregulation of GSNOR and its effect on protein S-nitrosation and other glutathione/-NO-mediated events is in its infancy. Further investigations into the role of GSNOR in health and disease are needed to reveal the most effective therapeutic options.

Disclosure statement

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