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Review

Mechanisms and targets of the modulatory action of S-nitrosoglutathione (GSNO) on inflammatory cytokines expression

Alessandro Corti [a,*], Maria Franzini [b,c], Ilenia Scataglini [a], Alfonso Pompella [a]

[a] Department of Translational Research and New Technologies in Medicine and Surgery, Medical School, University of Pisa, Italy
[b] Life Science Institute, Scuola Superiore Sant'Anna, Pisa, Italy
[c] Cardiology and Cardiovascular Medicine Division, Fondazione Toscana G. Monasterio – CNR, Pisa, Italy

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ABSTRACT

A number of experimental studies has documented that S-nitrosoglutathione (GSNO), the main endogenous low-molecular-weight S-nitrosothiol, can exert modulatory effects on inflammatory processes, thus supporting its potential employment in medicine for the treatment of important disease conditions. At molecular level, GSNO effects have been shown to modulate the activity of a series of transcription factors (notably NF-κB, AP-1, CREB and others) as well as other components of signal transduction chains (*e.g.* IKK-β, caspase 1, calpain and others), resulting in the modulation of several cytokines and chemokines expression (TNFα, IL-1β, IFN-γ, IL-4, IL-8, RANTES, MCP-1 and others). Results reported to date are however not univocal, and a single main mechanism of action for the observed anti-inflammatory effects of GSNO has not been identified. Conflicting observations can be explained by differences among the various cell types studies as to the relative abundance of enzymes in charge of GSNO metabolism (GSNO reductase, γ-glutamyltransferase, protein disulfide isomerase and others), as well as by variables associated with the individual experimental models employed. Altogether, anti-inflammatory properties of GSNO seem however to prevail, and exploration of the therapeutic potential of GSNO and analogues appears therefore warranted.

Introduction

S-Nitrosoglutathione (GSNO) is the endogenous S-nitrosylated derivative of the major antioxidant glutathione (GSH). In the past few years a large number of studies focused on GSNO properties – *i.e.* synthesis, transport across cell membranes and biological activities – and roles of GSNO as a store of nitric oxide (NO)[1] or as a component of NO-dependent signal transduction pathways were proposed [1]. In this perspective, a number of recent reviews comprehensively summarize the main findings in the field (*e.g.* [1–6]). As far as biological functions are concerned, one of the major areas of interest is the role of GSNO as modulator of inflammatory responses, with all the potential implications for GSNO-related therapies. Therapeutic potential of GSNO has indeed been already shown in several inflammation-related pathologic conditions, *e.g.* asthma [7], cystic fibrosis [8], undesired platelet aggregation and thromboembolism [9] and brain ischemia–reperfusion [10].

Different *in vitro* and *in vivo* models have been employed to investigate the modulatory role of GSNO on pro-inflammatory cytokines expression, but conflicting results have been reported, suggesting that several factors may influence the final outcome of GSNO treatment [11,12].

In the attempt to elucidate the mechanisms and the factors involved, different compounds – such as organic nitrates (*e.g.* glyceryl trinitrate), sodium nitroprusside (SNP), sydnonimines (*e.g.* SIN-1), S-nitrosothiols (*e.g.* S-nitrosoglutathione, S-nitroso-N-acetyl-DL-penicillamine) and NONOates (*e.g.* DETA-NONOate) – have been employed. Unluckily these compounds are known to decay with different mechanisms and with different kinetics [13,14], thus making comparisons difficult. An additional

* Corresponding author. Address: Department of Translational Research and New Technologies in Medicine and Surgery, Medical School, University of Pisa, Via Roma, 55, 56126 Pisa, Italy. Fax: +39 050 2218557.
E-mail address: alessandro.corti@med.unipi.it (A. Corti).

[1] *Abbreviations used:* SNP, sodium nitroprusside; NOS, nitric oxide synthases; PBMCs, peripheral blood mononuclear cells; SNOs, S-nitrosothiols; GSNOR, GSNO reductase; TrxR, thioredoxin reductase; NO, nitric oxide; cAMP-dep., effects observed are cAMP dependent; cGMP-ind., effects observed are not cGMP dependent; DCs, dendritic cells; GGT, gamma-glutamyltransferase; HSVEC, human saphenous vein endothelial cells; MSU, monosodium urate crystals; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PI3K, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate; PLCγ1, phospholipase Cγ1.

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confounding factor resulting from the administration of a NO donor is its interaction with the tightly regulated endogenous production of NO by nitric oxide synthases (NOS). Several studies focused on the effects of inhibition/activation of NOS on cytokines expression and inflammatory response [12,15,16].

Although with some discrepancies related to the analytical method used [17,18], GSNO was found at nano- to low micromolar concentrations in extracellular fluids and tissues [17,19–21], and, due to its nature of endogenous compound, it has been considered an "attractive candidate" for GSNO-based therapies [8]. The aim of the present review is to highlight some of the factors affecting the experimental use of exogenous GSNO as modulator of inflammatory cytokines expression and to discuss the inconsistencies reported in literature.

GSNO and inflammatory cytokines: lessons from *in vitro* models

A selection of studies performed *in vitro* with GSNO are summarized in Table 1. The most commonly used models were by far endothelial cells and peripheral blood mononuclear cells (PBMCs). The majority of the works dealt with a selected group of cytokines and chemokines, such as TNF-α, IL-1β, IL-6 and IL-8 [22–39], with only a few studies focusing on different chemokines such as IP-10 or MCP-1 [29,31,40], IL-2 [40–42], M-CSF [43], IL-4 [32], TGF-β1 [44] and IL-17 [45].

The effects produced by GSNO are quite heterogenous, with either a positive or a negative modulation of the same type of cytokine (*i.e.* TNF-α, IL-6, IL-8). When results are compared on the basis of cell types used, GSNO shows to exert an anti-inflammatory effect on endothelial cells [23,25,28], whereas conflicting results were obtained with respect to PBMCs and keratinocytes. The limited number of studies, however, does not allow to outline any particular cell specificity. It is conceivable that GSNO may differentially modulate cytokines expression in different cell types, also depending on both the tissue and the species (*e.g.* human, rat, mouse; [46]). Another major issue of such studies is related to GSNO concentrations used, ranging from as low as <10 micromolar up to $\geqslant$1 mM, *i.e.* concentrations even a hundred-fold higher than those usually detected in biological fluids. As a consequence, these data could help us elucidate the effects and the cellular targets of *pharmacological* – rather than *physiological* – GSNO concentrations [47].

In a small number of studies [22,35,38,39,43,44], the effects produced by exogenously added GSNO were also compared with those obtained by modulating endogenous NO production, mainly through NOS induction or inhibition. However a specific evaluation of endogenous levels of GSNO upon NOS modulation was not performed. In a study on a human mast cell line, the effects produced by eNOS induction upon IFN-γ treatment were mimicked by NO donors [35], and similar results were obtained in a human prostate cancer cell line transfected with iNOS [44]. NOS inhibitors such as N^G-amino-L-homoarginine (NAHA) [22], N^G-methyl-L-arginine (L-NMA) [43,44], N^G-monomethyl-L-arginine (L-NMMA) [38] or N^G-nitro-L-arginine methyl ester (L-NAME) [35] were also used. In other studies on murine macrophages, GSNO inhibited IL-1β secretion, whereas iNOS silencing by siRNA, its inhibition by L-NMMA [38] or the use of macrophage from mice lacking iNOS (*iNos−/−*) produced opposite effects [39].

Nevertheless it is conceivable that several factors – other than GSNO concentration – are likely to contribute to the final outcome of GSNO treatments.

GSNO decomposition

The half-life of the various S-nitrosothiols (SNOs) currently used varies dramatically depending on the biological system in which they are used [1]. S-nitrosothiols are subject to several mechanisms of decomposition, including S/N bond homolysis, photolytic S/N bond cleavage, metal ion-catalyzed decomposition, and hydrolysis [1]. As compared to other S-nitrosothiols, GSNO is quite stable – nevertheless, different non-enzymatic as well as enzymatic pathways were shown to promote GSNO degradation.

From a chemical point of view, GSNO can react with other biological molecules such as thiols (*e.g.* GSH, Cys), ascorbate, iron and copper ions. Nevertheless, in the cellular context the predominant reaction is supposed to be the enzymatic reduction of GSNO (see [1] for references). However it cannot be excluded that different culture conditions/incubation mixtures may influence GSNO stability or differentially address its final effects after an enzymatic reduction [48].

Different enzymes were shown to promote GSNO decomposition (Fig. 1). GSNO reductase (GSNOR; also known as alcohol dehydrogenase class III or glutathione-dependent formaldehyde dehydrogenase) is a NADH-dependent member of the alcohol dehydrogenase family. Studies *in vitro* demonstrated that GSNO is reduced by GSNOR using NADH as a cofactor to produce an intermediate (GSNHOH) which can either react with GSH to produce glutathione disulfide (GSSG) and hydroxylamine (NH_2OH) or rearrange to glutathione sulfinamide. Subsequently glutathione sulfinamide can spontaneously hydrolyze to produce glutathione sulfinic acid (GSO_2H) and ammonia (NH_3) (see Fig. 1) [49–51]. Several studies have established an important role for GSNOR in signal transduction by nitric oxide and protection against cytostatic or cytotoxic effects resulting from pathophysiological levels of protein S-nitrosylation [52,53]. It has been proposed that the homeostasis of endogenous NO/GSNO/SNO resulting from double gate-controlled modulation by synthases (NOS) and GSNOR might be critical in immune responses [4]. Indeed GSNOR is the only enzyme that has been demonstrated to regulate S-nitrosothiols levels *in vivo*, and connections between GSNOR activity and human disease have been suggested (see [1,5] for references). In this perspective, GSNOR has been proposed as a target for therapy in some pathological conditions such as lung and cardiovascular disorders, asthma and other inflammatory diseases [1].

γ-Glutamyltranspeptidase (GGT) is a plasma membrane enzyme that extracellularly hydrolizes GSNO to the less stable S-nitroso-cysteinylglycine (CGSNO) which rapidly releases NO in presence of trace metal ions [48,54]. It was suggested that further cleavage of CGSNO by a peptidase or transnitrosylation of a L-cysteine (L-Cys) residue can eventually produce S-nitroso-L-cysteine (CysNO; Fig. 1) [55,56]. CysNO can rapidly transfer SNO moiety into cells through the L-amino acid transporter system (L-AT) located on the plasma membrane [2,5]. CGSNO too could readily be imported into the cell by a dipeptide permease, a member of the ABC transporter family – as suggested by early studies on *Salmonella typhimurium* [57]. Nevertheless, other studies on RAW 264.7 cells demonstrated that extracellular GSNO can mediate intracellular S-nitrosothiol formation only when co-incubated with specific low molecular weight thiols [56]. In particular, incubations with GSNO and L-cysteine, L-cystine or D/L-homocysteine are able to increase intracellular S-nitrosothiol formation, whereas incubations with GSNO and L-cysteinyl-glycine do not [56]. Nevertheless it can be envisaged that by cleaving GSNO, GGT may play a role in facilitating SNO uptake and S-nitrosylation of intracellular proteins [2,48]. In this regard, it was demonstrated that GSNO-induced Akt kinase activity and the subsequent downstream HIF-1α stabilization are blocked by acivicin, a GGT inhibitor [58]. Similarly, acivicin prevents the effects of GSNO on 5-lipoxygenase expression in A549 cell line [59], whereas a GGT dependent GSNO catabolism differentially regulates IL-8 expression in IB3-1 cell line [37]. Interestingly, GGT expression levels vary considerably among cells types [60] and it may increase upon cell activation [42,61,62].

Table 1
GSNO effects on cytokines production in *in vitro* models.

Cellular model	Stimulus	GSNO concentration	Cytokines modulated*	Other factors modulated	Pathways studied	References
Human PBMCs, T cells	PHA	25 μM	- IL-2 - IL2R	↓↓ DNA synthesis	↑ cGMP	[41]
Human melanoma cell line G361	–	100–300 μM	↑↑ IL-8	–	↑ NF-κB, ↑ NF-IL-6 - AP-1	[22]
HSVEC	IL-1α IL-1β IL-4 TNF-α LPS	30 μM–1 mM	↓↓ IL-6 ↓ IL-8	↓ VCAM-1 ↓ ICAM-1 ↓ E-selectin	↓ NF-κB cGMP-ind.	[23]
HSVEC	TNFα oxLDL	200–500 μM	↓↓ M-CSF	–	↓ NF-κB ↑ IκBα ∼ AP-1 - GATA2 - GATA3 cGMP-ind.	[43,106]
Human neutrophils	LPS	0–2 mM	↑↑ IL-8	- Cell viability	cGMP-ind.	[24]
Human T and B cell lymphoma; peripheral blood T cells	– Anti-human CD3	50 μM–1 mM	↑ IL-2 - IL-2	↓↓ DNA synthesis	–	[42]
Human endothelial cell line ECV304	TNF-α	100 μM–1 mM	↓↓ IL-8	↓↓ Migration of neutrophils	↓ NF-κB ↑ IκBα	[25]
KB and HaCaT human keratinocyte cell lines	IL-1β	100–300 μM	↑ IL-8	–	–	[26]
Human monocytic U937 cells	PMA	500 μM	↑ TNF-α	–	↓ Sp1 cAMP-dep.	[27]
Human endothelial cell line ECV304	TNF-α	250 μM–1 mM	↓ IL-8	–	↓ AP-1 ↓ JNK	[28]
Primary human keratinocytes	IFN-γ TNF-α Serum-free medium	100 μM–2.5 mM	↓↓ IP-10 ↓↓ RANTES ↓ MCP-1 - IL-8	↓ ICAM-1	↓ NF-κB ↓ STAT-1 - AP-1	[29]
Human monocytic U937 cells	–	300–500 μM	↑↑ IL-8	–	Role of: OH· p38 NF-κB PI3K	[30]
Human monocyte-derived DCs	LPS CD40 ligand	0–2.5 mM	↑↑ IFN-γ ↓↓ IL-5 ↓↓ IL-10 ↑ TNF-α ↓↓ IL-12 p40 - IL-12 p70 ↓ IP-10 ↓ RANTES - IL-8	↓ CD86 - CD80 - CD83 - MHC class I and II	–	[31]
Human PBMCs	PHA IL-2	250 μM	↓↓/↑ IL-2 ↓ IL-13 ↓ IFN-γ - IL-4 - Lymphotactin - RANTES - IP-10 - MIP-1α - MIP-1β - MCP-1 - IL-8	↓↓ Cell proliferation - CD3 - CD25	–	[40]
Rat RBL-2H3 and mouse BMMC mast cells	IgE/Ag	125–500 μM	↓↓ IL-4 ↓↓ IL-6 ↓↓TNF-α	↓ Degranulation	↓ AP-1 ↓ PLCγ1 ↓ NF-AT - NF-κB - CREB - MAPKs	[32]
Human monocytes	TNF-α	0.0005–1 μM	↑↑ TNF-α	↑↑ IRAK-M - Cell viability	–	[33]
Human THP-1 cells and primary human monocytes	LPS	50–500 μM	↑↑ IL-8 ↑ TNF-α	–	↑ p38 - AP-1 - NF-IL-6 - NF-kB - Oct-1 cAMP-ind. cGMP-ind. cAMP-dep. cGMP-ind.	[34]

Table 1 (*continued*)

Cellular model	Stimulus	GSNO concentration	Cytokines modulated*	Other factors modulated	Pathways studied	References
Human mast cell line HMC1	IFN-γ and PMA	100 μM	↓ IL-8 ↓ CCL1	–	–	[35]
Human prostate cancer cells PC-3MM2, LNCaP and DU145	Serum-free media	50 μM-1 mM	↓ TGF-β1	–	–	[44]
Mouse CD3 positive T cells	CD3/28 antibodies	1–400 μM	↓↓ IL-17 - IL-4	–	↓ STAT3 ↓ RORγ - STAT4 - STAT6 - T-bet - GATA3	[45]
Human PBMCs	–	<10 μM	↑ IL-6	–	↑ NF-κB cGMP-dep.	[36]
	–	>100 μM	↓ IL-6	–	↓ NF-κB cGMP-dep.	
Human bronchial epithelium IB3–1cells	LPS	300 μM	↑ IL-8	↑ Cell proliferation	∼ NF-κB ↑ p38 - ERK1/2 - JNK	[37]
	LPS + GGT		↓↓ IL-8	↓ Cell proliferation	↓ NF-κB ↓ ERK1/2 ↑ p38 - JNK	
Murine peritoneal macrophages; human THP-1 cells; human PBMCs	LPS ATP Nigericin MSU	100 μM–1 mM	↓↓ IL-1β - IL-6 - TNF-α	↓↓ Caspase-1	↓ NLRP3	[38]
Mouse macrophages	Pam3CSK4	125–500 μM	↓↓ IL-1β - TNF-α	↓↓ Caspase-1	↓ NLRP3 cGMP-ind.	[39]

Abbreviations and symbols used: cAMP-dep. = effects observed are cAMP dependent; cGMP-ind. = effects observed are not cGMP dependent; DCs = dendritic cells; GGT = gamma-glutamyltransferase; HSVEC = human saphenous vein endothelial cells; MSU = monosodium urate crystals; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; PI3K = phosphoinositide 3-kinase; PMA = phorbol 12-myristate 13-acetate; PLCγ1 = phospholipase Cγ1; ↑:"increase of"; ↓:"decrease of"; ↑↑:"increase ⩾ 100%"; ↓↓:"decrease ⩾ 50%"; -:"no effects on" ∼: "slight variation".

* Data reported are referred to modulation of mRNA expression, protein or both.

Other GSNO degrading enzymes are the NADPH-dependent carbonyl reductase 1 (CBR1) and the thioredoxin system. The former shows some similarities of reaction products and stoichiometry with GSNOR (see Fig. 1) [63,64]. The latter is one of the major players in the reduction of low-molecular weight and protein S-nitrosothiols and is involved in both denitrosylation and transnitrosylation reactions. The proposed mechanism implies that the reaction occurs through transnitrosylation of thioredoxin (Trx) and release of GSH. Then nitroxyl (HNO) is released and oxidized Trx is finally reduced by thioredoxin reductase (TrxR) and NADPH (Fig. 1) [65].

In addition, other enzymes such as cell surface protein disulfide isomerase (PDI) [66–68], copper- and zinc-containing superoxide dismutase (CuZnSOD) [69,70], xanthine oxidase [71,72] and glutathione peroxidase 1 (GPx1) [73] have been suggested to affect GSNO metabolism/degradation [1] (Fig. 1). The catalytic mechanism proposed for PDI involves a nitroxyl disulfide intermediate which, after undergoing a one-electron oxidation, decomposes to yield NO plus dithiyl radical. The final product after one enzymatic turnover is an oxidized protein active site and NO [68]. CuZnSOD was suggested to catalyze the reductive cleavage of GSNO. The mechanism involves GSH and the redox turnover of copper in CuZnSOD, with the latter serving as a highly efficient catalyst of GSNO breakdown [70]. GSH would reduce enzyme associated Cu^{2+} to Cu^{1+} which in turn would mediate the reductive decomposition of GSNO. CuZnSOD – but not MnSOD [69] - would thus function *in vitro* as a NO S-transferase, catalyzing NO transfer from GSNO to protein targets [70]. Also xanthine oxidase (XO) – in the presence of purine (hypoxanthine, xanthine) substrates – was demonstrated to induce GSNO decomposition. The proposed mechanism involves a superoxide anion-dependent reduction of GSNO to yield NO, which in turn reacts with a second superoxide anion molecule to yield peroxynitrite [71,72]. Finally GSNO decomposition by selenium-containing GPx1 was also described. The proposed mechanism is based on the diselenides-catalyzed formation of NO from S-nitrosothiols in the presence of thiols. The diselenide (R′Se-SeR′) reacts with the free thiol (RSH) to produce the selenosulfide (RS-SeR′) and the selenol (R′SeH). The selenol generated reacts with RS-NO to release NO and a disulfide (RS-SR) [73].

Against this background, it is conceivable that different cell types may express different levels of the above mentioned enzymes, and that experimental conditions may modify such expression. For instance, it is known that (a) GGT is induced upon exposure of cells to oxidative stress or cytokines (see [62] for references) and that (b) different levels of GGT can be detected in incubation media depending on the final FCS concentrations added.

GSNO concentrations, incubation times, co-administration of other agents

Part of the inconsistencies observed upon GSNO treatments likely just depend on the activation status of cells, *i.e.* the presence of other stimulating/inhibiting conditions or compounds, such as LPS, TNF-α and IFN-γ (Table 1). The employment of serum-free media [29,44,74] is also a critical point in many studies, as cellular susceptibility towards NO largely dependent on the pre-activating stimuli [75]. The absence of serum may in fact produce a cell "priming" due to starvation [76]. On the other hand, the presence of serum during incubations may also have consequences due both to S-nitrosothiols reaction with serum protein thiols [2] and to the presence of GSNO degrading enzymes (*e.g.* GGT).

Factors such as GSNO concentration and length of incubation are also likely to influence significantly the final outcome. A

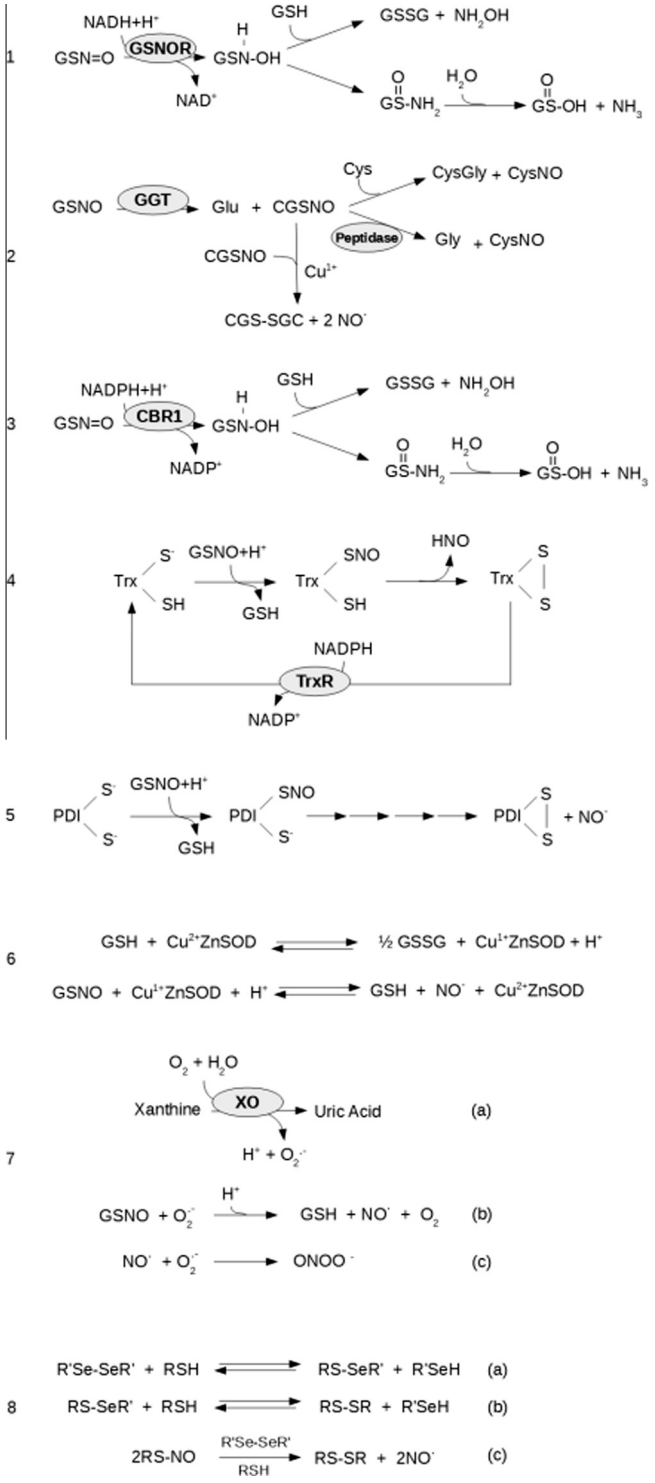

Fig. 1. Proposed mechanisms for the enzymatic decomposition of GSNO. Schematic representation of GSNO decomposition by (1) GSNO reductase (GSNOR) [49,148], (2) γ-glutamyltranspeptidase (GGT) [48,149], (3) carbonyl reductase 1 (CBR1) [63], (4) thioredoxin system (Trx/TrxR) [65,150], (5) protein disulfide isomerase (PDI) [68], (6) copper- and zinc-containing superoxide dismutase (CuZnSOD) [70], (7) xanthine oxidase (XO) [71,72] and (8) glutathione peroxidase 1 (GPx1) [73]. See text for more details.

"biphasic", concentration-dependent activity was indeed proposed for different NO-donors [77,78], including GSNO. Low, "physiological" GSNO concentrations (<10 μM) were found to increase the nuclear specificity proteins (Sp)3-dependent transcription, while inhibiting Sp1 binding to DNA in A549 cells. Conversely, higher GSNO concentrations (10–100 μM, as those associated with inflammation and nitrosative stress) promoted opposite effects [47]. It was proposed that physiological levels of GSNO may promote physiological homoeostasis, whereas nitrosative stress levels may signal a change from the expression of housekeeping genes to the expression of stress-response genes [47]. At variance, Sp1/Sp3-dependent expression of 5-lipoxygenase (5-LO) was shown to be increased at low (<1 μM) and inhibited at higher (>5 μM) GSNO concentrations, respectively [59]. Similarly, NF-κB activation and IL-6 expression were promoted at lower (<10 μM) and decreased at higher (>100 μM) GSNO concentrations [36]. A biphasic effect was also demonstrated for HIF-1α activation/accumulation, 100 μM GSNO being able to attenuate $CoCl_2$-induced HIF-1α stabilization and 1 mM GSNO being ineffective [79]. Finally, an alleged "triphasic" GSNO-dependent PPARγ activation – remarkable at 100–200 μM but virtually absent at doses lower than 50 μM or higher than 500 μM – was also described [80].

It is likely that these quite paradoxical concentration-dependent effects might reflect the different sensitivity of cellular targets to GSNO. This behavior is not a specific property of GSNO: similar biphasic responses are known to be produced, *e.g.* by redox regulation of factors such as NF-κB. Indeed, the nuclear translocation of NF-κB requires an oxidative environment – capable of favouring the degradation of IκB – whereas its binding to the DNA requires a reducing environment. High levels of oxidative stress may impair the nuclear redox status, thus hampering NF-κB DNA binding [81,82]. Notably, part of the targets known to be modulated by S-nitrosothiols is represented by factors involved in cell signaling, including ubiquitylation, thus supporting its broad pleiotropic influence on protein post-translational modification [83]. Interestingly, the enhanced Sp3–DNA binding in the presence of physiological concentrations of GSNO is associated with increased Sp3 expression, which in turn may partially result from the inhibition of Sp3 ubiquitination and proteosomal degradation. At levels of GSNO associated with nitrosative stress, Sp3 binding is shut off, at least partially through inhibition of Sp3 expression [47].

Despite the growing number of reports outlining novel protein targets for S-nitrosothiols, there is a limited number of studies focusing on the levels of the S-nitroso species found in cells or tissues under physiological or pathological conditions [84].

As stated above, the greater part of published *in vitro* studies – including those reported in Table 1 – employed GSNO concentrations that are higher than those detectable *in vivo* in resting conditions [8,11]. As already mentioned, it cannot be excluded that such GSNO concentrations are reached under pathological or pharmacological conditions [47].

Another critical point is certainly the timing of GSNO administration – relative to that of other compounds. In a study on human PBMCs, preincubation of cells with GSNO for 48 h before PHA exposure strongly inhibited IL-2 release, whereas simultaneous addition of both compounds significantly enhanced it [40]. Similarly, extended pre-incubation times (24–48 h) with a NO donor were shown to be required in mast cells for inhibition of IgE/Ag-induced cytokines expression and degranulation [32,85,86].

Finally, experimental conditions used may also dramatically influence the final outcome. In early papers (*e.g.* [87]) GSNO was used in combination with superoxide dismutase added as a "protecting" agent, *i.e.* to prevent superoxide from binding to and inactivating NO. However, CuZnSOD was also proposed to function *in vitro* as an NO S-transferase, catalyzing NO transfer from GSNO to protein targets [70].

Lastly, additional variability can derive from the oxygen concentration during incubations. GSNO has in fact been reported to promote HIF-1α stabilization under normoxia [88,89] while inhibiting it under hypoxia (see below for more details) [90,91].

GSNO: how does it work?

The mechanisms of GSNO formation *in vitro*, have been studied in some detail, and some explanations have been proposed, *e.g.* direct reaction of NO/nitrosylating species with GSH or formation of protein/low molecular weight S-nitrosothiols followed by subsequent transnitrosation to GSH [1,92]. Nevertheless, our knowledge of the mechanisms of S-nitrosothiol formation *in vivo* is still incomplete [84]. Similarly, the mechanisms by which GSNO can influence cellular responses have also been extensively studied. In a recent review, Broniowska and colleagues [1] outline the fact that most of the studies employing GSNO were interpreted according to the wrong assumption that GSNO mainly works by liberating NO. Actually several factors – as summarized above – may profoundly affect the specific mechanism of GSNO decay. Moreover it is worth remarking that biochemical changes occurring after cell exposure to S-nitrosothiols (with respect to thiol chemistry) are different from those observed upon NO exposure [56].

A first issue is related to GSNO uptake. As stated above, it was demonstrated that GSNO itself cannot enter directly into cells and that the predominant mechanism involved requires the transfer of the nitroso group from GSNO to the thiol group of extracellular L-Cys. The CysNO thus formed was shown to be readily transported into cells by means of the L-amino acid transporter system (L-AT), and to S-nitrosylate other low molecular weight thiols (mainly GSH) or protein thiols intracellularly [1,56]. It is conceivable that the redox state of the cell may influence extracellular L-Cys availability, which in turn is supposed to derive from extracellular cystine through the sequence *cystine uptake–intracellular reduction–cysteine release* [56]. Likewise, specific experimental conditions, such as the composition/integration of incubation media, can substantially influence L-Cys availability [5,56,93,94].

Other mechanisms involving the delivery of NO or its oxidation products have been described [1,84], and it is interesting to note that this route is also subject to differential modulation. For instance, it was demonstrated that cholesterol content in membranes contributes to the spatial heterogeneity of NO diffusion and signalling, as the diffusion rate of NO across membranes is inversely related to the cholesterol content [95].

A second issue is how GSNO can modulate cellular responses. It was observed that S-nitrosothiols may mimic many of the downstream effects of NO, but through potentially disparate intracellular mechanisms [56]. Moreover the effects of exogenous S-nitrosothiols on intracellular S-nitrosylation have little to share with the cell biology of NO [2]. As regard GSNO in particular, two additional mechanisms of action were proposed, *i.e.* protein S-nitrosylation and protein S-glutathionylation, besides canonical NO-dependent pathways [1,84,96].

Protein S-nitrosylation

S-nitrosylation – the covalent attachment of NO to the sulfur moiety of cysteine – is a post-translational, redox sensitive, reversible modification. It is known that its target cysteine residues are highly tissue- and context-specific, and that their S-nitrosylation is dynamically regulated both in synthesis and degradation [1,5]. Probably the most important reaction of a S-nitrosothiol inside a cell or in a biological fluid is transnitrosylation, *i.e.* the reversible transfer of the S-nitroso functional group to a receiving thiolate compound [84]. A large number of proteins has been shown to undergo S-nitrosylation and among them some trascriptional regulators and upstream intermediates involved in cells signaling [6]. S-nitrosylation leads to changes in protein activity, protein–protein interactions or subcellular location of target proteins, and is in many ways analogous to phosphorylation [3]. The picture is further complicated by the fact that modulation of cell signals by S-nitrosylation often affects factors involved in other major mechanisms of post-translational modification, such as phosphorylation, acetylation or ubiquitination [83].

S-nitrosylation is primarily regulated enzymatically, with NOSs and additional enzymes (*e.g.* GSNOR, thioredoxin) capable of performing S-nitrosylation and denitrosylation reactions [3,96,97]. S-nitrosylation thus meets the requirements of a regulatory post-translational modification, including specificity, reversibility and enzymatic control [15], although – at variance with phosphorylation – is not strictly dependent on enzymes for its formation and breakage [96]. Exposure of cells to exogenous S-nitrosothiols is therefore likely to interfere with this tightly regulated mechanism.

As reported in Table 1, major transcription factors such as NF-κB, AP-1 and HIF-1α – involved in cytokines expression – were shown to be modulated by GSNO. A recent review by Sha and Marshall [6] exhaustively summarized how S-nitrosylation regulates gene transcription as well as the multiple targets involved.

Nuclear factor κB (NF-κB) is a transcriptional factor regulated through complex interactions of IκB family of inhibitory proteins and IκB kinases-mediated phosphorylation events. Studies on NF-κB S-nitrosylation were performed with different NO-donors such as SNP, SNAP, or S-nitrosocysteine in both cellular and cell-free systems. DNA binding of NF-κB was shown to be inhibited by S-nitrosylation of a specific cysteine on the p50 monomer [98–100] as well as by S-nitrosylation [101] or Tyr-nitrosylation [102] on the p65 monomer. As far as GSNO is concerned, in endothelial cells TNF-α mediated activation of NF-κB was shown to involve denitrosylation of p65, while GSNO mediated inhibition was associated with S-nitrosylation of the same monomer [103]. This suggests that the net activation of NF-κB in endothelial cells during inflammation could depend on a balance between stimulatory and inhibitory factors, and that constitutive NO production may play a role in tonically inhibiting NF-κB activation under basal conditions [43]. Others have shown that IKKβ – the catalytic subunit of IKK complex required for NF-κB activation – may also undergo S-nitrosylation upon exposure to GSNO and L-Cys, thus resulting in NF-κB inhibition. Again, TNF-α stimulation induced a rapid denitrosylation and activation of IKK [104]. Conversely, in a study with an ovalbumin model of allergic airway disease in mice, GSNO induced S-nitrosylation of NF-κB p65 but did not affect IKKβ [105]. In endothelial cells and neutrophils, GSNO was demonstrated to stabilize the NF-κB inhibitory protein IκB-α by preventing its degradation and/or by increasing its expression [25,106–108]. On the contrary, other studies showed no involvement of NF-κB upon GSNO exposure [32,34], whereas its activation was instead shown in a small number of cases [22,36,80]. An indirect effect of GSNO on cytokines expression can be mediated through the NF-κB dependent expression of cyclooxygenase-2 (Cox-2) and the consequent prostaglandin biosynthesis, although conflicting reports also exist on this point [80,109].

Activating protein (AP)-1 is a heterodimeric transcription factor belonging to the basic domain leucine zipper (bZIP) family, consisting – in its most active form – of the two subunits Fos and Jun. Conflicting results are reported as regard GSNO effects on DNA binding of AP-1, oscillating between inhibition [28,32,106] and induction [80,110]. The mechanisms proposed are either dependent on cGMP [110] or based on down-regulation of c-Jun N-terminal kinase (JNK) [28]. However, NO-donors such as SNP or SIN-1 were shown to promote direct S-nitrosylation of Jun and Fos, thus inhibiting DNA binding of AP-1 [111,112].

Hypoxia-inducible factor-1 (HIF-1), the transcription factor regulating the expression of several genes involved in hypoxic tolerance and inflammation, is a heterodimer composed of two subunits: the regulatory subunit HIF-1α and the constitutively expressed HIF-1β. In hypoxia, HIF-1α is stabilized through decreased activity of prolyl hydroxylases that target the protein for degradation. Under normoxia, exposure to GSNO can result in

the accumulation of HIF-1α protein (and its concomitant DNA binding) through distinct mechanisms, *i.e.* a phosphatidylinositol 3′-kinase (PI3K)–Akt-dependent pathway [89,113] or an increased expression of both HIF-1α and HIF-1β [88], possibly depending on S-nitrosylation, which is in fact known to increase HIF-1 activity by modifying HIF-1α at two distinct cysteine residues [114,115]. GSNO also prevents HIF-1α degradation by inhibiting its ubiquitination. Indeed, in human embryonic kidney cells under normoxic conditions, GSNO was shown to inhibit HIF-1α prolyl hydroxylase (PHD) activity in a dose-dependent fashion, thus inhibiting the subsequent interaction of HIF-1α with von Hippel Lindau protein (pVHL) [116]. Conversely, other authors demonstrated that GSNO can promote HIF-1α inhibition under hypoxia, possibly through PHD activation [90,117,118]. The mechanism modulating PHD activity is supposed to be driven by NO interaction with a ferrous iron ligand located within the PHD active site [116].

Other signal transduction pathways have been shown to be affected by GSNO. In a study on U937 human myelomonocytic cells, it has been demonstrated that also Sp1 and Sp3 transcription factors are intracellular targets of GSNO [27]. GSNO concentrations <10 μM were shown to increase Sp3 and to inhibit Sp1 DNA binding, respectively. On the other hand, higher GSNO levels resulted in a complete reversal of the observed effects [47]. It is conceivable that these might be mediated by the S-nitrosylation of cysteine residues in Sp3 and Sp1 specific domains [119].

Components belonging to the Ras and JNK pathways were also shown to be modulated. GSNO can S-nitrosylate Ras [120] with possible consequences on downstream pathways including ERK1/2, Jak-STAT, and phosphoinositide 3-kinase (PI3K)–Akt. GSNO was indeed shown to modulate the activity of PI3K–Akt and PTEN [58,113,121]. Other targets of GSNO are mitogen-activated protein kinase (MAPKs), JNK [28,122], ERK1/2 [37,80] and p38 [34,37]. The latter is implicated in the stabilization of mRNAs containing AU-rich elements in their 3′-untranslated regions, as is the case with major cytokines TNFα, IL-1β, IL-6 and IL-8 [123].

Other targets of GSNO modulation are elements operating upstream of NF-κB, AP-1 or MAPKs. Elements in Toll-like receptors (TLRs) and NOD-like receptors (NLRs) signaling were also shown to be modulated by S-nitrosylation. TLRs are the central innate immune sensors for a broad array of pathogen- and damage-associated molecular patterns (PAMPs; DAMPs) and upon ligand binding they initiate signaling cascades leading to the activation of transcription factors such as AP-1, NF-κB and interferon regulatory factors (IRFs) [124]. With the exception of TLR3, all TLRs utilize a MyD88-dependent pathway resulting in the production of TNF-α, IL-1, IL-6 and other cytokines dependent on NF-κB; they also trigger MAPKs cascades that lead to activation of AP-1 and cyclic AMP response element-binding protein (CREB). MyD88 is an adaptor protein recruited upon receptors activation. It was observed that a specific S-nitrosylation within MyD88 Toll/interleukin-1 receptor homology (TIR) domain resulted in a slight reduction of NF-κB activation [125].

Several cytokines that play important functions during development, tissue modeling and/or immune responses are known to be subject to posttranslational processing and control. Activation of TLRs pathway leads to the transcription of the pro-IL-1β gene, but the subsequent processing of the precursor peptide into its active form is mediated by multi-protein complexes termed 'inflammasomes'. These hetero-oligomeric structures consist of the IL-1β processing enzyme caspase-1, the adapter protein ASC, and a sensor protein of the NLR (NOD-like receptor) or ALR (AIM2-like receptor) families. Among others, the NLRP3 inflammasome is a multi-protein complex that triggers the maturation of the pro-inflammatory cytokines IL-1β and IL-18 [126]. Interestingly, it was demonstrated that GSNO dramatically inhibited IL-1β secretion from murine peritoneal macrophages in a dose-dependent manner, whereas IL-6 or TNF-α secretion was not affected [38]. Similarly, in a model of *Mycobacterium tuberculosis*-infected mouse macrophages, GSNO and SNAP were shown to inhibit the processing and release of IL-1β (but not TNFα) as efficiently as IFN-γ [39]. Authors demonstrated that NO can promote S-nitrosylation of both NLRP3 and caspase-1, but only NLRP3 S-nitrosylation appears to inhibit the assembly of the NLRP3 inflammasome and IL-1β release [39]. These pieces of evidence were paralleled by the observation that endogenous NO derived from iNOS also negatively regulated NLRP3 inflammasome activation [38,39].

In this complex regulatory mechanism, factors like CREB and caspase 1 may also be modulated by S-nitrosylation [6,38,39,127].

Another route through which GSNO may modulate cytokines expression involves S-nitrosylation of cysteine protease calpain. Calpain was shown to be inhibited by S-nitrosylation of its active site thiol group, resulting in a down-regulation of mast cells adhesion to fibronectin [128]. Interestingly, calpain inhibition was also demonstrated to downregulate Th1/Th17 inflammatory cytokines and mRNA in PBMCs of multiple sclerosis patients [129].

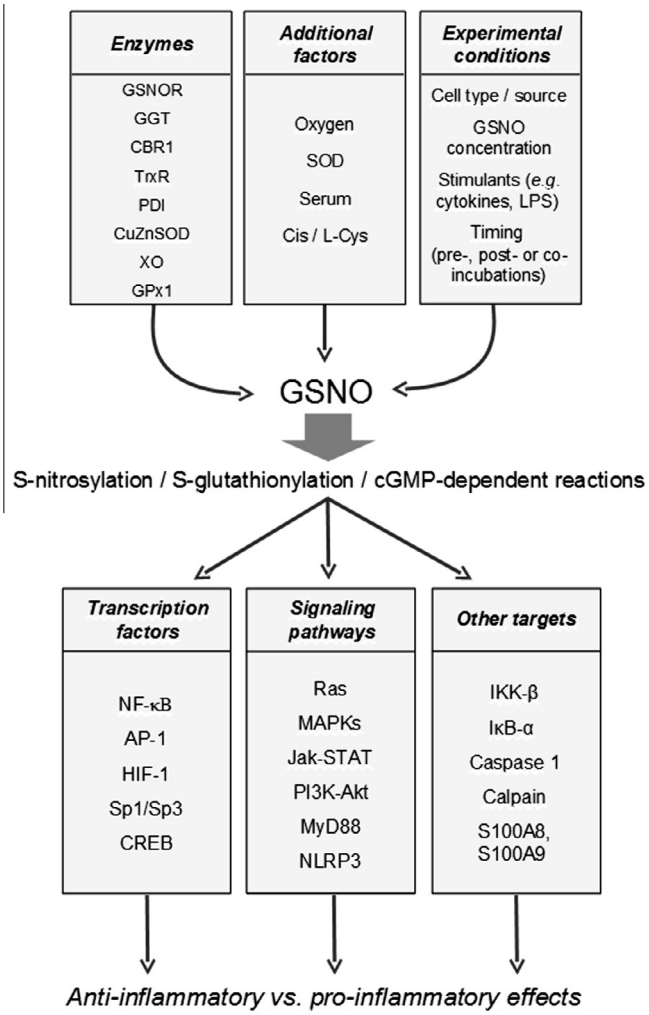

Fig. 2. Schematic representation of targets modulated by GSNO dependent reactions. GSNO has been shown to modulate inflammatory processes by affecting the activation status of a series of transcription factors and other components of signal transduction chains. Results to date are however not univocal and part of these inconsistencies can be explained by differences among cell types as to the relative abundance of enzymes active in GSNO metabolism, as well as by variation of conditions in different experimental models.

Table 2
GSNO effects on inflammation in *in vivo* models.

Model	Disease	GSNO treatment	GSNO treatment effects	References
Rat	Chronic cerebral hypoperfusion	50 μg/kg/day 2 months	↑↑ Learning and memory (day 4–6) *Brain tissue* ↓ ICAM-1/VCAM-1 ↓ amyloid-β (Aβ) *Brain Endothelial cell culture* ↓ NF-κB, ↓ STAT3 ↓ ICAM-1, ↓ VCAM-1 *Rat primary neurone cell* ↓ β-secretase activity	[137]
Rat	Focal cerebral ischemia	1 mg/kg body weight jugular vein cannulation at the time of reperfusion	↓↓ infarction area ↑ cerebral blood flow *Brain tissue* ↓ TNF-α, ↓IL-1β ↓ iNOS ↓ microglia/macrophage (ED1, CD11-b) ↓ leukocyte function-associated antigen-1 ↓ intercellular adhesion molecule-1 ↓↓ caspase-3 *Rat primary astrocytes cell & microglial cell line BV2* ↓ NF-κB ↓ iNOS	[138]
Rat	Controlled cortical impact (CCI)	50 μg/kg body weight orally 2 h following CCI, and repeated daily	↑↑ motor function (14 days) ↑ neurological score (6 days) ↑↑ sensory dysfunction (>7 days) *Brain tissue* ↓ ICAM-1 ↓ microglia/macrophage (ED1) ↓ MMP-9 ↓ iNOS ↓ apoptosis ↓ blood brain barrier leakage and edema ↑ ZO-1, ↑ occludin	[139]
Rat	Controlled cortical impact (CCI)	50 μg/kg body weight orally 2 h following CCI, and repeated daily	Brain tissue ↓ inflammatory infiltration ↓↓ ICAM-1 mRNA ↓ 3-nitrotyrosine (3-NT), ↓ 4-HNE ↓ blood brain barrier leakage and edema ↓↓ brain infarction area ↓ loss of myelin and damage to axons ↑ total and reduced GSH *plasma* ↓ ONOO−, ↓ TBARS, ↓ 3-NT	[140]
Mice	Experimental autoimmune encephalomyelitis (EAE)	0.5 or 1 mg/kg, oral administration daily from day 0 (prophylaxis) or when mice had visible EAE symptoms	↓ EAE symptoms *Spinal cord* ↓↓ $CD4^+$ infiltration *Treated animals splenocytes* ↓↓ IL-17 - IFN-γ and IL4 production	[45]
Mice	Cerebral Malaria	3.5, 0.35, 0.035 mg/mice, IP, twice a day from day 0 to day 8	↓ parasite growth (3.5 mg/mice) ↓↓ leukocytes in brain vessels ↓↓ brain haemorrhages (3.5 mg/mice) ↑ GSH	[141]
Rat	Lumbar spinal stenosis	50 μg/kg body weight 1 h after the injury, and daily thereafter.	↓ locomotor dysfunction ↑ pain threshold *Spinal cord* ↓ demyelination	[142]

(*continued on next page*)

Table 2 (*continued*)

Model	Disease	GSNO treatment	GSNO treatment effects	References
			↓↓ cellular infiltration ↓ apoptosis *nerve fibers* ↑ endothelial VEGF	
Mice	Ovalbumin-induced Allergic airway disease	500 nmol oropharyngeal administration 30 min before OVA challenge	*Lung tissue* ↓ HIF-1 activation by hypoxia ↓ NF-κB activation ↑ S-nitrosylation of p65 - increased cellular infiltration - increased mRNA of IL-13, MUC5ac, GOB5, iNOS *Bronchoalveolar lavage* - increased cell counts - increased protein levels Plasma - increased OVA-specific IgE	[105]
Mice	Experimental autoimmune uveitis	1 mg/kg/day 14 days	*Retina* ↑ retinal protection ↓ mRNA for TNFα, ↓↓ mRNA for IL-1β, IFN-γ ↓↓ mRNA for IL-10 - IL-4, - IL-6 *Splenocytes* ↓ T-cell proliferation ↓↓ secretion of TNF-α, ↓IFN-γ ↓↓ secretion of IL-10.	[143]
Mice	Conditional ablation of enteric glia cells	10 mg/kg/day, intraperitoneal administration	*In vivo* ↓ intestinal permeability *Intestinal tissue* ↓↓ TNF-α mRNA *Caco-2 cell line* ↑ ZO-1 expression ↑ F-actin expression	[144]
Rat	Experimental periodontitis	25, 100, 500 nmol intragingival injections 1 h before periodontitis induction, and thereafter, daily for 11 days.	*Periodontium* ↓↓ alveolar bone loss (100 nmol) ↓ MPO activity ↓ MMP-1, ↓ MMP-8 ↓ iNOS ↓ NF-κB ↓↓ IL-1β (100 nmol) ↓ TNF-α ↓↓ malondialdehyde (100 nmol) ↑ GSH (100 nmol) ↑↑ bone alkaline phosphatase (100 nmol)	[145]
Human keratino-cytes	Psoriasis	14-day 1% GSNO ointment	*Biopsy of psoriatic lesion* ↓ IP-10, MCP-1, RANTES - IL-8 ↓ ICAM-1 ↓ $CD14^+$ and $CD3^+$ infiltration *Keratinocytes primary cell culture* ↓ IP-10 ↓↓ MACP-1, RANTES - IL-8 ↓ ICAM-1 and sICAM-1	[29]

Symbols used: ↑:"increase of"; ↓:"decrease of"; ↑↑:"increase ⩾ 100%"; ↓↓:"decrease ⩾ 50%"; -:"no effects on".

S-glutathionylation

S-glutathionylation, *i.e.* the reversible formation of protein-glutathione mixed disulfides, has been demonstrated to result from GSNO exposure [130]. It has been suggested that such modification could arise either from a direct reaction between reduced proteins and GSNO or as a result of denitrosylation of S-nitrosylated proteins by reduced GSH [131]. Some proteins seem to be preferentially glutathionylated by GSNO, with the preference for S-nitrosylation or S-thiolation possibly deriving from tuning of reaction kinetics by the thiol environment [1]. Trascriptional factors such as the AP-1 component Jun and NF-κB monomer p50 [132,133] or regulatory proteins S100A8 and S100A9 [134] were suggested to undergo S-glutathionylation on conserved cysteine residues upon GSNO exposure. Nevertheless the question of

whether S-thiolation is an important cellular response to S-nitrosothiol formation or exposure has not yet been conclusively addressed [1].

Other regulatory mechanism

Finally, other studies have demonstrated the occurrence of GSNO effects based on more canonical, NO-related mechanisms [1]. Classical cGMP-dependent effects were observed in PBMCs [36,41], rodent fibroblast and epithelial cell lines [110], as well as in mouse BV-2 microglial cells [74]. The role of cGMP-dependent protein kinase (PKG) in the activation of NF-κB by direct phosphorylation of p65 or IκB [36] or in the activation of CREB [74] has been proposed.

It is worth noting that pathways potentially modulated by GSNO may be differentially activated depending on GSNO decay, cell type, and cell activation status. Some target proteins may be preferentially activated by specific modifications (S-nitrosylation or S-thiolation), and the susceptibility of the modified proteins to denitrosylation and deglutathionylation reactions may also be different [135]. A recent study identified sites that would be preferably transnitrosylated by GSNO [136]. The opposing effects of GSNO reported in the literature are thus not necessarily conflicting, being highly context dependent. The following examples are rather clear in this perspective: (a) different kinetics of GSNO decomposition may increase or decrease IL-8 expression through p38 or ERK1/2 and NF-κB pathways, respectively [37]; (b) the cGMP-dependent signaling system is not efficient in the U937 cell line and, consequently, the redox-sensitive pathways may be expected to prevail in these cells [30]; (c) upregulation of protective mechanisms in response to non-lethal GSNO concentrations, LPS or cytokine prestimulation may redirect the ability of a subsequent GSNO exposure to upregulate p53 and to initiate macrophage apoptosis [75]. These mechanisms may explain, at least in part, the reported ability of GSNO to act in both a pro- and anti-inflammatory manner.

Further factors undergoing to modulation by GSNO or other S-nitrosothiols and NO-donors were reported in recent reviews (*e.g.* [6,12,15]). The present overview refers to studies focused on cytokine expression by exogenously added GSNO, and shows that such treatment may modulate multiple molecular targets involved in gene transcription ranging from transcription factors to upstream signaling cascades, mRNA stability and translation (Fig. 2). The large number of factors involved accounts for the growing number of genes regulated by GSNO treatments. Notably all mammalian iNOS genes exhibit homologies to binding sites for numerous transcription factors including AP-1, CREB, HIF, NF-κB, NF-IL6, Oct-1, Sp1 and STAT-1α [16], *i.e.* targets modulated by GSNO. The latter is thus supposed to exert a feedback regulation on the very NO production [104].

In vivo studies

To date, there have been nearly ten *in vivo* studies on animal models dealing with the effects of GSNO on inflammation; Table 2 reports a summary of the results obtained. Most investigations were concerned with neurological injury [45,137–142]. Other studies have described immunological derangements [105,143] or induction of inflammation in the intestine [144], periodontium [145] or uvea [143].

Despite the multiplicity of pathological contexts considered and the differences in routes of administration and doses, these studies collectively reported beneficial effects of GSNO treatment on disease symptoms and modulation of inflammation. GSNO administration was in fact associated with: (i) lower cell infiltration in the diseased tissue; (ii) lower activation of resident macrophages; (iii) lower expression of adhesion molecules (i.e. ICAM-1, VCAM-1); (iv) lower expression of matrix metalloproteases (MMP-1, MMP-8, MMP-9); (v) lower degrees of apoptosis (via inhibition of caspase 3); (vi) lower iNOS activity; (vii) decreased oxidative stress markers (mieloperoxidase activity, peroxynitrite, oxidized lipids and proteins). Regarding in particular the expression of cytokines, this aspect was taken into account by five studies. These latter [105,138,143–145] confirmed that NF-κB activation is reduced, as showed in *in vitro* studies, and also reported a decreased expression of the pro-inflammatory cytokines TNFα, IL-1β, IFN-γ and IL-13. IL-10 was also shown to be lowered by GSNO treatment [143]. On the other hand, Olson et al. [105] obtained different results in a study on allergic airway inflammation, showing that *in vivo* instillation of GSNO did not change significantly markers of inflammation or mucus metaplasia, even if NF-κB and HIF-1 activation were reduced. Authors concluded that the discrepancy might be due to the involvement of other not-investigated signalling pathway, in addition to NF-κB and HIF-1, and/or to C57BL/6 mice being less sensitive to NF-κB activity.

Interestingly, GSNO-dependent reduction of inflammation response in the periodontitis model [145] was observed with the lowest GSNO doses (25 and 100 mmol), while the intragingival injection of 500 mmol was not associated with reduction of neither inflammation (IL-1β, TNFα) nor oxidative stress markers (malondialdehyde, GSH). These findings confirm the bimodality of biological action of NO and NO-compounds, pointing out that likely a concentration threshold, affecting the control of inflammation, likely exists.

In humans, several clinical trials investigated the therapeutic efficacy of GSNO, but they have been mainly focused on GSNO effects on platelet aggregation, vasodilation and bronchodilation [1,146]. A small study conducted on three healthy human subjects and three psoriatic patients showed that a 14-day skin treatment with an ointment containing GSNO reduced the expression of chemokines as well as of adhesion molecules (Table 2) that promote the inflammatory infiltrate during psoriasis [29].

The cited studies did not deepen in exploring the effect of *in vivo* endogenous NO production on cytokine expression. Anyway studies on mice knock-out for the iNOS gene confirmed that endogenous NO modulate the expression of IL-1β [38,39].

Altogether, *in vivo* studies suggests that GSNO could be a promising therapeutic agent in several diseases, but further work is necessary to detail the mechanisms by which GSNO regulates inflammatory cells, endothelium, smooth muscle cells as well as platelets, and to identify the best application routes and doses to the scope.

Concluding remarks

GSNO plays an immunoregulatory role in the induction and inhibition of inflammatory cytokines. The effects of exogenous GSNO *in vitro* appears to be determined by several factors ranging from the cell type to the variables associated with incubations, with possible consequences on the route and kinetics of GSNO decay and on the reactions produced. The biological consequences of these reactions are diverse and not fully predictable, nevertheless, basing on data from *in vivo* models, anti-inflammatory properties of GSNO seem to prevail on pro-inflammatory ones. Again the responsivity of the animal model, the concentration and/or the route of GSNO administration used may significantly affect the final outcome. The many variables that can affect GSNO activity, and the finding that most of its biological functions are related to transnitrosylation reactions, may thus justify the design of novel GSNO-based derivatives for therapeutic use [147]. Further studies are however required for a full comprehension of all factors involved, and to ascertain the feasibility of GSNO-based pharmacological treatments for specific disease conditions.

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