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Commentary

Don't just say no: Differential pathways and pharmacological responses to diverse nitric oxide donors

Juan J. Chiesa, Fernando M. Baidanoff, Diego A. Golombek*

Laboratorio de Cronobiología, Universidad Nacional de Quilmes/CONICET, Argentina

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ABSTRACT

Nitric oxide (NO·) is a gaseous free radical molecule with a short half-life (~1 s), which can gain or lose an electron into three interchangeable redox-dependent forms, the radical (NO·), the nitrosonium cation (NO^+), and nitroxyl anion (HNO). NO· acts as an intra and extracellular signaling molecule regulating a wide range of functions in the cardiovascular, immune, and nervous system. NO· donors are collectively known by their ability to release NO· *in vitro* and *in vivo*, being proposed as therapeutic pharmacological tools for the treatment of several pathologies, such as cardiovascular disease. The highly reactive NO· molecule is easily oxidized under physiological conditions to N-oxides, nitrate/nitrite and nitrogen dioxide. Different cellular responses are triggered depending on: 1) NO· concentration [e.g., nanomolar for heme coordination in the allosteric site of guanylate cyclase (sGC) enzyme]; 2) the type of chemical bound to the nitrosated group (i.e., bound to nitrogen, N-nitro, or bound to sulphur atom, S-nitro) determining post-translational cysteine nitrosation; 3) the time-dependent availability of molecular targets. Classic NO· donors are: organic nitrates (e.g., nitroglycerin, or glyceryl trinitrate, GTN; isosorbide mononitrate, ISMN), diazeniumdiolates having a diolate group [or NONOates, e.g., 2-(N,N-diethylamino)-diazenolate-2-oxide], S-nitrosothiols (e.g., S-nitroso glutathione, GSNO; S-nitroso-N-acetylpenicillamine, SNAP) or the organic salt sodium nitroprusside (SNP). In addition, nitroxyl (HNO) donors such as Piloty's acid and Angeli's salt can also be considered. The specific NO· form released, as well as its differential reactivity to thiols, could act on different molecular targets and should be discussed in the context of: a) the type and amount of NO· species determining the sensitivity of molecular targets (e.g., heme coordination, or S-nitrosation); b) the cellular redox state that could gate different effects. Experimental designs should take special care when choosing which NO· donors to use, since different outcomes are to be expected. This article will comment recent findings regarding physiological responses involving NO· species and their pharmacological modulation with donor drugs, especially in the context of the photic transduction pathways at the hypothalamic circadian clock.

1. Introduction

Since the discovery of the endothelial generation of the free radical NO· regulating vascular tone in 1987 [1], this gaseous molecule was found to act as an autocrine and paracrine messenger signaling a wide range of physiological processes, including angiogenesis, platelet aggregation, inflammation, and neurotransmission. Indeed, a growing interest has emerged for the development of pharmacological tools with the ability to regulate NO· bioavailability, directed mainly for the treatment of cardiovascular disorders such as hypertension and angina pectoris. A hallmark for this development was the management of regulated NO· delivery to molecular targets through NO·-releasing compounds (i.e., NO· donors) that "liberate" this gaseous molecule under physiological conditions. NO· cellular transduction regulates effector proteins through diverse post-transcriptional modification mechanisms (i.e., activating soluble guanylate cyclase [sGC], S-nitrosation, or trans-S-nitrosation). Although several compounds have similar NO· donor ability, their different chemical structures generate differential pharmacological profiles. Due to NO· bioavailability, reactivity of nitrosated intermediates with thiol sulfhydryl groups forming S-nitroso compounds at cysteine residues may occur [2]. These additional targets can modify the activity of effector proteins, acting at the level of enzymes such as NO· synthase (NOS), channels such as the ryanodine receptor 2 (RyR2) [3] or, as discussed below, putative circadian clock proteins working as transcription factors. The redox environment of the cell determines the steady state concentration of NO·,

* Corresponding author at: Universidad Nacional de Quilmes/CONICET, Roque Saenz Peña 352, (1876) Bernal, Buenos Aires, Argentina.
E-mail address: dgolombek@unq.edu.ar (D.A. Golombek).

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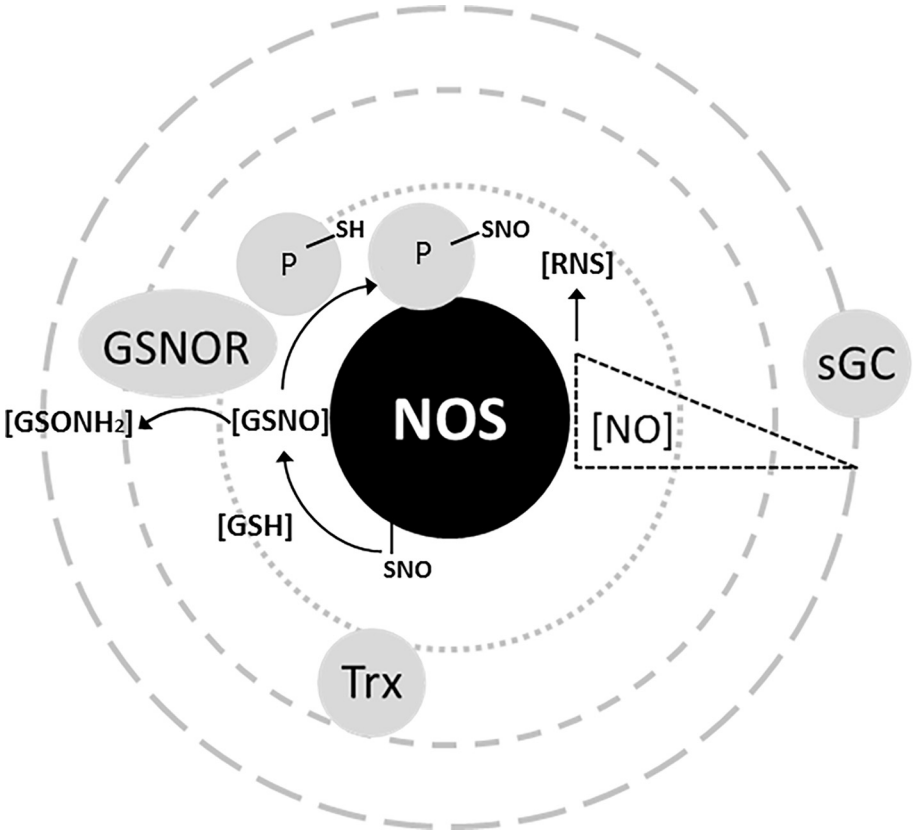

Fig. 1. Concentration range-dependent NO· signaling. A classic signal transduction pathway activates sGC through low, decreasing NO· concentrations far away from the NOS source. Short-ranged S-nitrosations (SNO) are limited to regions with increased NO·, i.e., close to NOS cellular location where N-oxides and radical nitric species (RNS) derivatives (e.g., N_2O_3, NO_2) are increased. SNOs of proteins (P) are formed in this short-range region through reaction with RNS, or trans-S-nitrosation with S-nitrosothiols [e.g., S-nitrosoglutathione (GSNO)]. Systems that regulate SNOs [e.g., thioredoxins (Trx) and GSNO reductase (GSNOR)] are located in cellular proximity to NOS.

since oxidant conditions will generate N-oxides (mainly nitrate and nitrite, NO_3^-, NO_2^-), while a reduced environment will favor the reaction with both glutathione (to form S-nitroso-glutathione, GSNO) and cysteines (generating S-nitroso-peptide). Moreover, the availability of cellular targets might change throughout the day, resulting in a chronopharmacological regulation of NO· activity. In addition, other compounds such as nitroxyl anion (NO^-, or HNO) have been demonstrated to exert physiological activity [4,5]. Which specific NO· donor is used, combined with the physiological state of the cell, will determine a different biochemical/pharmacokinetic profile for NO·, and differentially affect its putative molecular targets. This article will comment on recent findings regarding physiological responses involving NO· species and their pharmacological modulation with donor drugs, especially in the context of the photic transduction pathways at the hypothalamic circadian clock.

2. Regulation of NO biosynthesis and metabolism

The biosynthesis of NO· is catalyzed by the NOS heme oxide-reductase enzyme by oxidizing L-arginine, using molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates, and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4) as cofactors for electronic transference, generating L-citrulline. Vascular endothelium is the main source of NO·, with endothelial NOS isoform (eNOS) regulating smooth muscle tone and hemostasis (e.g., platelet aggregation). The inducible (iNOS) isoform generates NO· in several cell types such as macrophages, hepatocytes and synoviocytes, to regulate inflammation and immune functions [6]. Finally, the neuronal (nNOS) isoform generates NO· involved in neuronal communication [7,8], and was also shown to be present at cardiomyocytes regulating contractility [9]. Both eNOS and nNOS are constitutively expressed generating basal NO· concentration. iNOS is transcriptionally controlled and, in tandem with NADPH oxidase, generates high NO· and superoxide concentrations to form the unstable radical peroxinitrite ($ONOO^-$), with cytotoxin/cytostatic activity. Both eNOS and nNOS increase their activities responding to rising intracellular calcium concentration ($[Ca^{2+}]_i$), elicited by the transduction of mechanical stimulation sensed by endothelial cells [10], or by photic (glutamatergic) stimulation of the suprachiasmatic nuclei (SCN), the site of the mammalian circadian clock [11]. $[Ca^{2+}]_i$ transients bind to calmodulin (CaM), regulating its interaction with the CaM-binding region of NOS [12], increasing Ca^{2+}/CaM dependent protein kinase II (CaMKII) activity to phosphorylate NOS, inducing a conformational change that favors electronic transfer for reductase activity [13]. Indeed, phosphorylation/dephosphorylation by several kinases [e.g., CaMKs, cAMP dependent protein kinase (PKA), protein kinase B (Akt)] and phosphatases [e.g., protein phosphatase 1 (PP1), PP2B (calcineurin)], are key post-translational regulations of eNOS acting on specific phosphosites to increase or decrease its activity [14]. Other mechanisms indirectly regulate eNOS activity, such as myristoylation/palmitoylation that regulate the recruitment of eNOS at plasma membrane/subcellular locations, or the interaction with membrane microdomains through caveolae coat proteins caveolin-1 (Cav-1) [14]. Moreover, a non-classic pathway regulates NOS activity in response to cellular redox microenvironment, switching its activity from oxidase to reductase. This mechanism is mediated by the formation of S-nitrosothiols in a docking site for glutathione, close to a zinc tetra-thiolate region [15]. In that way, NO· can exert negative feedback on its own synthesis [16], and the dimeric S-nitroso enzyme can promote the synthesis of S-nitroso-glutathione (GSNO) through a trans-S-nitrosation mechanism [2].

It is important to note that for physiological transduction – either at vascular endothelium or neuronal tissues – picomolar to nanomolar NO· are required [17]. At higher concentrations, other reactions become important, especially considering the actual O_2 cellular concentration needed to form nitrosating species able to regulate protein and cellular functions (Fig. 1). The half-life of this gaseous messenger will depend on the rate of generation/consumption/degradation determined mainly

by NO· auto-oxidation by O_2 levels [18]. NO· is easily oxidized to up to eight different N-oxides in aqueous solution, with nitrates (NO_3^-) and nitrites (NO_2^-) being the major breakdown end-products. The kinetics of NO· auto-oxidation predict a half-life of 1 h at physiological O_2 concentration, due to the slow reaction of NO· with O_2 superoxide to form peroxinitrite ($ONOO^-$), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and then NO_3^- and NO_2^-. NO· also reacts with transition metals in heme groups to form metal-nitrosyl adducts. High-affinity, irreversible reaction with ferrous heme groups of oxyhemoglobin and myoglobin generate rapid (in the order of seconds) inactivation of NO·. The affinity for heme groups of mitochondrial chain complexes lowering cellular respiration (also with negative feedback by $ONOO^-$, and S-nitrosothiols) seems to be an adaptive response to hypoxia [19]. As a general view, after activation of biosynthesis at physiological conditions, NO· can act as a transient local messenger, without a complex metabolism for clearance.

2.1. NO· as messenger is not the only message

NO· is the compound to which physiological activity is attributed. It is generated by several cell types acting as a highly regulated autocrine and paracrine signaling molecule. Due to its non-polar and highly reactive nature, it can diffuse in gradients of up to ~100 μm from its source, with a half-life in the range of seconds. These features allow the target tissue to respond proportionally to NO· concentration, e.g., to generate point-by-point vasodilation in response to turbulence in microvascular beds [10], to regulate synaptic plasticity [20], even acting as a retrograde messenger in local neuronal networks. High concentrations of NO· interacting with high-affinity heme groups in hydrophobic subcellular microenvironments (e.g., mitochondrial, or plasma membrane folds) are efficient to promote enzyme regulation [e.g., cytochrome C [19]]. Low (nanomolar) concentrations, diffusing through aqueous cytosolic compartment (majorly converted to NO_3), are enough for heme-coordination of soluble enzymes, such as sGC [21]. Importantly, NO· can oxide to N-oxide intermediates favoring a reaction with thiols and generating nitrosothiols on regulatory cysteines of effector proteins [2].

Reduction of NO·, isoelectronic with molecular O_2, generates NO^-, which is the conjugated base of nitroxyl (HNO, or azanone), a highly reactive molecule that auto-dimerizes to N_2O and reacts with oxygen, nucleophiles, and transition metals at metalloporphyrins. HNO exhibits distinct chemical and biological properties from those of NO·, and its endogenous source remains unclear [22]. In the last years it was possible to detect physiological levels of HNO, regulating vascular tone [4] and cardiac output [23] through calcitonin gene-related peptide (CGRP) and transient receptor potential channel 1 (TRPV1) signaling. Its vasoprotective effects rely on vasodilation induction, platelet aggregation inhibition, and limitation of vascular smooth muscle cell proliferation [24]. HNO has some overlapping properties with NO·, including reactivity toward some ferrous heme proteins such as sGC [25]. But a key property that distinguishes HNO from NO· is its ability to directly interact with and oxidize thiols and thiol-containing proteins. As such, HNO can directly target RyR2 [26] and sarco/endoplamsic reticulum Ca^{2+} ATPase (SERCA) [27] to increase $[Ca^{2+}]_i$ cycling and contractility in the myocardium. The scenery for NO transduction involves interactions with other gasotransmitters, such as hydrogen sulfide and carbon monoxide regulating vascular tone [4,28] and gastroprotection [29,30].

As an example of the differential effects of chemically distinct NO· donors, we shall propose their differential pathways at the circadian clock, due to the redox cellular state effecting reactivity on targets, and/or their distinct pharmacokinetic profile and bioavailability. NO· donors can generate a predictable dose-effect if acting through specific targets (i.e., heme coordination by NO· levels regulating sGC activity), but also can induce pleiotropic, S-nitrosation transduction likely through other nitrosative messengers such as GSNO. Indeed, the so-called "redox transduction" must consider the S-nitroso-proteome generated by incorporation of NO moieties on regulatory cysteines (leading to the formation of protein S-nitrosothiols).

3. Molecular targets of NO·

The canonical cellular receptor for NO· in vascular tissue is the heme group of sGC enzyme. Its activation by transient nanomolar NO· levels increase the conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), leading both to restoring $[Ca^{+2}]_i$ in the sarcoplasmic reticulum, and to the activation of the type I c-GMP-dependent protein kinase (PKG). Endothelial-derived NO· reduces smooth muscle cells contraction through PKG phosphorylation of the myosin-binding subunit of the myosin light chain phosphatase, which in turn dephosphorylates myosin II light chain [31]. PKG also phosphorylates and inhibits Rho kinase activity, reducing the propensity for vasoconstriction and promoting vasodilation [32]. NO· donors are also effective for promoting vasodilation of coronaries, but also by increasing contractility of cardiomyocytes by β-adrenergic-, CaMKII- and nNOS-induced NO· levels [3]. This is accomplished by non-classic (i.e., independent of cGMP) redox-dependent S-nitrosation and aperture of sarcoplasmic reticulum channel RyR2, and of other components regulating the $[Ca^{+2}]_i$ cycle [33]. This reversible, post-translational modification in the RyR increasing $[Ca^{+2}]_i$, also operates for synaptic plasticity in cerebellar Purkinje cells, depending on endogenous NO· levels [34].

4. The NO· pathway in the circadian clock

The NO·/cGMP pathway has also a key role signaling the photic synchronization of the circadian clock in suprachiasmatic (SCN) neurons, through intrinsically photosensitive retinal ganglion cells projecting glutamatergic afferents to the hypothalamus [11]. The SCN clock responds differentially to a brief (i.e., 15 min) light exposure under constant darkness delivered at different circadian times. In most nocturnal rodents, these light-pulses delivered when the animal is awake (i.e., the circadian "subjective" night) generate phase-shifts of the circadian clock. When the stimulation is delivered at the early subjective night (i.e., close to the behavioral activity onset), there is an induced delay of the circadian phase, while if applied at the late night, it generates a phase-advance of the clock. This bi-phasic effect of light can be explained by taken a pathway bifurcation for delays or advances downstream of NO· messenger. Photic $[Ca^{2+}]_i$-CaM activation of nNOS is elicited at both early and late subjective night [7]. The NO·/cGMP pathway is specifically required for phase-advances, due to necessary NO· heme coordination in the sGC allosteric site, increasing cGMP levels and cGMP-dependent protein kinase II (PKGII) activity [35]. In contrast to the well-known cardiovascular pathways for NO·, photic downstream substrates are still unknown in the SCN neurons. For phase-delays, the aperture of endoplasmic reticulum RyR channel leading to $[Ca^{2+}]_i$ increase is involved [36]. The circadian expression of RyR in the early subjective night [37], or of PKGII in the late subjective night [38] is a possible explanation for the pathway bifurcation. However, other hypothesis should be tested, including how circadian changes in redox state changes modulate nNOS activity, generating different messengers [(i.e., NO·, NO_2, S-nitroso glutathione (GSNO)]. In addition, NO· levels could differentially enhance classic (NO/cGMP/PKG) or non-classic transduction through S-nitrosation of regulatory cysteines in the pathway and/or effector components (see the following section). Moreover, as already stated, donor drugs could be either acting simply as NO· releasers or generating S-nitrosations through their differential thiol reactivity. Through any of this mechanisms, phase-delaying and phase-advancing pathways must converge at the level of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), whose phosphorylated version is the canonical activator of genes for the photic synchronization of the circadian clock

[39].

5. S-nitrosation transduction

Nitrosation reactions represent the conversion of organic compounds (e.g., RS-, RO-, RN-) into nitroso derivatives (R-NO). S-nitrosation is the addition of a nitroso group by condensation of a thiol (R-SH) and nitrous acid (NO^+) by one-electron oxidation of NO·, generating an S-nitrosothiol (RSNO). The biochemical mechanisms proposed to generate nitrosothiols are: 1) NO auto-oxidation generating nitrosative derivatives (N_2O_3, NO_2), having high affinity for thiols; 2) thyil radical recombination; 3) transition-metal based oxidation (important in mitochondrial electron chain transport); 4) binding of low molecular weight thiols (S-thionylation, in particular S-glutathionylation, S-cysteinylglycinylation, and S-cysteinylation); 5) transference of NO· by trans-S-nitrosation, either from S-nitroso groups, or protein-to protein interactions [2]. This transient, reversible post-translational modification, depends on high NO· levels [40,41] and it is proposed as a short-range mechanism in which target proteins must be close to NO· source (i.e., colocalization with nNOS, at least in cardiomyocytes [42]). Also, hydrophobic compartments favor direct S-nitrosation [43] likely by increasing partition (and stabilizing) of O_2 and NO·, but access to solvent of cysteine residues was seen to favor the thiolate ($R\text{-}S^-$) reactant group. Indeed, modelling S-nitrosation has not predicted any consensus site likely due to the various mechanisms involved in nitrosothiol generation [2]. Martinez-Ruiz et al. [41] proposed a model in which substrate proximity is necessary for trans-S-nitrosation reactions, which can also occur in the aqueous cytosolic compartment [44]. This is determined by cellular NO· levels (i.e., subcellular proximity to nNOS) and/or availability of S-nitrosothiols such as GSNO [45] (Fig. 1). Although this model was initially proposed for cardiomyocytes, some of the potential effects of NO· donors in the SCN neurons can be predicted due to similar transduction pathways through different targets. Moreover, it is suggested that the formation/degradation of small S-nitrosothiols [(GSNO and S-nitrosocysteine (CysNO)] may represent a mechanism for storage and transport of NO· [2]. Protein-to-protein interactions finely regulate global S-nitrosations of surface cysteines, which is also modulated by de-S-nitrosating enzymatic systems involved in the homeostasis of the redox state, such as thioredoxins (TRX), glutathione (GSH) peroxidase, and GSNO reductase (GSNOR) [2].

It is now clear that S-nitrosation signaling is of importance in physiological regulation, such as cardiovascular [46] and neuronal function [47]. S-nitrosation of proteins typically results in a conformational change that modifies enzymatic activity, protein-protein interactions, or cellular localization [48]. Excessive generation of reactive oxygen/nitrogen species (ROS/RNS), specially $ONOO^-$, cause depletion of sulfhydryl groups and oxidation of relevant proteins occurring in several chronic pathological states, such as hypertension and neurodegeneration [49 47]. They involve dysregulated inflammatory pathways due to altered redox transduction, generating high ROS/RNS levels, endoplasmic reticulum stress [50], aberrant protein misfolding and accumulation (e.g., Tau and β amyloid in neurodegenerative disorders), and/or loss of effector functionality. For instance, dysregulated S-nitrosation of RyR was found to be related to an altered sarcoplasmic Ca^{2+} leak and arrhythmogenicity in cardiomiocytes [42]. Excitotoxicity found in neurodegeneration is attributed to excessive neuronal NO·-induced $[Ca^{2+}]_i$ release by dysregulation of RyR1 S-nitrosation [51]. It is proposed that desensitization of sGC to NO· by S-nitrosation is part of the vascular endothelial dysfunction due to oxidative/nitrosative stress in chronic hypertension, and of the mechanism of tolerance to nitrate drugs [52]. Indeed, de-S-nitrosating antioxidant agents, such as N-acetylcysteine (NAC) were proposed as new pharmacological tools for neurodegenerative disorders [53,47]. It should be taken that at physiological conditions, the S-nitroso bound has inherent lability and propensity to spontaneously react with thiols and generate disulphide bounds. It is argued that this modification should not be taken as a generalized end post-translational effector, but a transient, redox intermediate in the formation of disulphide bounds stabilizing protein structure [54]. This idea is of importance if redox cellular derivatives (e.g., ROS/RNS, GSH, and/or GSNO) are taken as messengers changing the structure, half-life, and degradation of core circadian clock proteins, as will be discussed below.

6. The type of NO· donor affects its outcome

Since NO· is easily oxidized to N-oxides, molecular carriers of NO (i.e., NO· donor drugs) were designed to stabilize the radical until its release is required. Molecules having the general X–N═O motif are direct NO· donors, while indirect donors (e.g., nitrates, nitrites, metal-NO complexes, and furoxans) need to be enzymatically metabolized [55]. Also, the NO·-biosynthesis precursor L-arginine increases vascular NO· bioavailability for treatment of endothelial dysfunction [56] and gastric ulcer [57]. The ones most used in clinical settings are nitrovasodilators, i.e., drugs that generate vascular dilation by NO·-induced smooth muscle relaxation. Organic nitrates (e.g., nitroglycerin or glyceryl trinitrate, GTN; isosorbide mononitrate, ISMN), and the organic salt sodium nitroprusside (SNP) were used for treatment of angina pectoris and hypertensive crisis, but clinically limited as they induce tolerance and systemic hypotension [58]. Their pharmacological profiles depend on the oxidation state of the nitrogen that is ultimately reduced to NO· in the physiological compartments: while SNP gains one electron, three are required in organic nitrates as GTN, with the aid of mitochondrial enzyme aldehyde reductase-2 [59]. Amine-based diazeniumdiolates having a diolate group [or NONOates, e.g., 2-(N,N-diethylamino)-diazenolate-2-oxide, DEA-NO, or 2,2′-(Hydroxynitrosohydrazino)bis-ethanamine, DETA-NO], are also used as donors, with the advantage over organic nitrates of spontaneous NO· release, which prevents metabolic tolerance [59,60]. Nebulized NONOates are efficient to reduce pulmonary vascular resistance and mean arterial pressure, without affecting systemic blood pressure and cardiac output in patients with acute respiratory distress syndrome [61]. S-nitrosothiols such as tert-dodecane, S-nitroso-N-acetylpenicillamine (SNAP), and GSNO, have been demonstrated to be useful for controlled NO· release for vascular relaxation [62] and activation of sGC enzyme in the skin [63]. S-nitrosothiols are also effective to generate trans-S-nitrosation of proteins such as RyR2 [3] and SERCA regulating $[Ca^{2+}]_i$ cycle [64] in cardiomyocytes. N-nitroso-tryptophan derivatives such as N-nitrosomelatonin (NOMel) and N-acetyl-N-nitroso-tryptophan (NANT) also show the ability to increase NO· levels *in vitro* [65,66] and *in vivo* [67], and are proposed also to generate S-nitrosothiols [68].

Chemically diverse donors have demonstrated the common ability to release NO· at vascular and neuronal tissues, usually taken as a unique messenger for specific targeting. However, this should be taken as a simplistic framework. For instance, SNP, SNAP and DEA-NO exhibit different potencies for raising cGMP in chromaffine cells [69], where the effective concentrations and kinetics of NO· released could be very different. In other words, the effect of different donors could be very different from the effect of endogenous, low concentration and transient NO· biosynthesis by eNOS/nNOS activity. The need for reductive metabolism (i.e., reductants bioavailability) for NO· generation by SNP or GTN, or the reactivity of NO^+ moiety of SNP with protein amines and/or thiol residues (i.e., NO· accumulation), generates non-additive effects on NO· concentrations, without reflecting the endogenous eNOS/nNOS biosynthesis [70]. Increasing NO· levels signal the circadian activity of L-type voltage-gated Ca^{2+} channels in retinal cones through a NO/cGMP/PKG pathway [71]. However, for these experiments SNAP and SNP were used as donors, which can generate NO· through very different pathways [55]. Several S-nitrosating NO· donors (GSNO, SNAP, and SNP) showed the same ability for persistent inhibition of vascular contraction in aortic rings, while this was not observed for DEA-NO and GTN [72]. The extent of NO· released by S-

nitrosothiols depends on the type of thiol, and the concentration of transition metals [70]. In addition, NO· release by these compounds is favored by a previous trans-S-nitrosation. These donors are useful for mimicking a biological response where nitrosation reactions predominate, but less effective for classic NO· signaling involving eNOS/nNOS activation [73]. NANT and NOMel generate similar NO· modulation of hypoxia-inducible factor 1 *in vitro*, but dissimilar to GSNO [66]. Thus, an *a priori* similar S-nitrosation and/or NO· donor ability for these compounds should not be considered. NONOates are taken as effective for mimicking transient eNOS/nNOS biosynthesis of NO·, since its decomposition rate is only dependent on the amine component (e.g., DEA-NO half-life, 2–4 min vs. DETA-NO, 20 h), pH and temperature [70]. These different rates of NO· generation make NONOates useful to mimic the low, transient burst of NO· synthetized by eNOS/nNOS (or high, tonic NO· generated by iNOS).

Nitroxyl (HNO) donors emerged as new pharmacological agents [25] due to their differential chemical and biological properties as compared to NO·. The most well-known HNO donor is obtained by decomposition in slightly alkaline aqueous solution of trioxodinitrate sodium salt ($Na_2N_2O_3$), also known as Angeli's salt (AS), which generates HNO and a minor proportion of NO· and NO^- [74]. Cardiovascular properties, promoting lusitropic and ionotropic (without chronotropic) effects increasing cardiac output, as well as vasodilating properties of AS, have been demonstrated in several *in vitro* and *in vivo* models and are partially mediated by sGC-dependent signaling [75]. Other HNO donors are Piloty's acid, a sulfonamide derivative that releases HNO under basic conditions, and isopropyl amine NONOate.

7. NO· donors at the photic transduction pathway

We hypothesized that increasing NO· levels at the circadian clock level would potentiate the transduction of photic-induced phase-shifts. In standard chronobiological protocols analyzing locomotor rhythms of hamsters, we have studied the central, intracerebroventricular effects of chemically different compounds with NO·-donor ability: SNAP [76], N-nitrosomelatonin (NOMel) [67], GSNO, and the nitroxyl (HNO) donor Angeli's salt (AS) [77]. While SNAP and GSNO enhanced both light-induced phase advances and delays of the circadian phase, NOMel only potentiated phase advances (failing even at high doses to potentiate delays). Moreover, NOMel generated a similar increase of NO· (i.e., NO_3^-/NO_2^-) levels in the SCN neurons when administered during the early or the late circadian night. In addition, extracellular scavenging of NO· inhibited both paracrine action and photic information influx in the SCN only for phase-advances [8]. This suggests that increased NO· levels by NOMel at the SCN (and likely SNAP and GSNO) work as a gaseous messenger in the pathway for light-induced phase advances, through sGC/cGMP/PKG activation. Since we had previously demonstrated that photic delays are independent of increased NO· levels, a short-range, non-classic pathway could be involved. SNAP and GSNO might behave both as effective donors for sGC activation [78,63] generating phase advances, as well as trans-S-nitrosating compounds to promote RyR aperture [64] and, in turn, phase delays.

Considering its *a priori* ability for trans-S-nitrosation *in vitro* [79,68], increased NO· levels by NOMel should have been effective to enhance photic RyR aperture and thus delays. But its pharmacological effect on the circadian clock *in vivo* seems to depend on its ability to liberate gaseous NO· specifically for inducing phase advances [67]. One could consider that an N-nitroso compound such as NOMel might exhibit a theoretically less thiophilicity when compared with both SNAP and GSNO. These are low molecular weight S-nitrosothiols having nitro groups bound to the sulphur atom that are easily transferred to thiols of target cysteines [80]. In addition, the different pharmacokinetic profiles of the donors used and/or the differential sensitivity to the pharmacological NO· levels, should also be considered.

We have also studied the effects of Angeli's salt (AS), an HNO donor, in the photic circadian pathways [77]. Light-induced phase advances of locomotor rhythms were enhanced by this compound, whose ability to interact with sGC was previously demonstrated [81,5]. However, it is not clear whether this effect is related to a direct HNO binding to the sGC heme group, or if the compound must be previously converted to NO· [82]. In addition, differential reactivity of NO· vs. HNO against metals (ferric vs. ferrous) and cysteines (thiols vs. thiolates) [25] should be taken, as occurs in the cardiovascular system [83]. Increasing HNO did not affect circadian phase delays, despite its ability to promote RyR2 aperture in cardiomyocytes [26]. Moreover, an increased glutathione/glutathione disulfide (GSH/GSSG) ratio could have a role decreasing HNO levels [83] in the early subjective night. Most of the evidences on the mechanism of action of AS are *in vitro*; however, the evidences *in vivo* are scant, mainly due to the failure in the detection of physiological HNO levels. Still, the increase of HNO at the SCN was detected electrochemically [84] with the pharmacological intracerebroventricular doses used. Therefore, even considering the high theoretical nucleophile activity of HNO, it is possible that thiolates in cysteine targets of RyR2 are not available in the early subjective night (when light pulses induce phase delays of the circadian system). However, since RyR2 can suffer trans-S-nitrosation, the enhancing effects of GSNO and SNAP at this time could be explained by a different thiol oxidation by S-nitroso compounds. In addition, thiol regulation of redox state can also alter circadian rhythms.

7.1. Circadian clock interactions with the redox state

Circadian clock oscillation in most mammalian cells is sustained by the activity of transcriptional/translational interacting feedback loops [85]. The canonical *clock* and *bmal1* (or *arntl1*) genes activity increase CLOCK and BMAL1 protein levels. These basic helix–loop–helix (bHLH) transcription factors, which contain PAS domains, can heterodimerize to activate *per1-2* and *cry1-2* genes. In turn, period (PER) and cryptochrome (CRY) proteins form heterodimers that inhibit the activity of *clock* and *bmal1*, re-setting the circadian molecular clock to the initial state. The CLOCK:BMAL1 heterodimer also activates the orphan nuclear receptor *rev-erb α/β* y *ror α/β* genes, generating an additional feedback loop which reinforces the oscillation. Photic transduction leads to the transcriptional regulation of both *per1* and *per2* genes [39], therefore re-setting the circadian phase.

The term "redox state" (i.e. the intracellular redox potential) broadly describes the balance between reduced and oxidized proteins within cells, which in turn is determined by the level of generation and buffering of cellular ROS/RNS. Circadian cellular oscillations in the redox homeostasis system were found in erythrocytes, independent of the canonical circadian molecular clock [86]. In turn, the circadian system generates a myriad of physiological outputs to generate predictive homeostasis in the timing of processes regulating energy metabolism [87]. Thus, it is not surprising that redox oscillations interact with the circadian clock components at several levels [88]. For example, NAD(H)/NAD^+ and NADP(H)/$NADP^+$ ratios enhance/reduce DNA binding of the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers [89]. The oxidized forms inhibit NAD^+-dependent deacetylase sirtuin-1 binds to BMAL1:CLOCK to regulate the circadian genes *bmal1*, *per2*, and *cry1* [90], and redox disulphide-thiol sensors of heme groups alter REV-ERBα/β activity [91].

It is also interesting that other compounds, both endogenous and in pharmacological administration, affect redox state and might interact with the nitrergic pathway. Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthetized and liberated mainly by the pineal gland, through circadian and photoperiodic regulation by an SCN polysynaptic output relaying at the superior cervical sympathetic ganglion. A wide evidence suggests its antioxidant properties reducing inflammation in several tissues, acting as RNS/ROS scavenger [92], or through the regulation of prooxidant iNOS gene expression and enzyme activity (Fan et al., 2018, Life Sci). Melatonin can also reduce basal eNOS/nNOS activity in the hypothalamus and, in particular, in the SCN during the

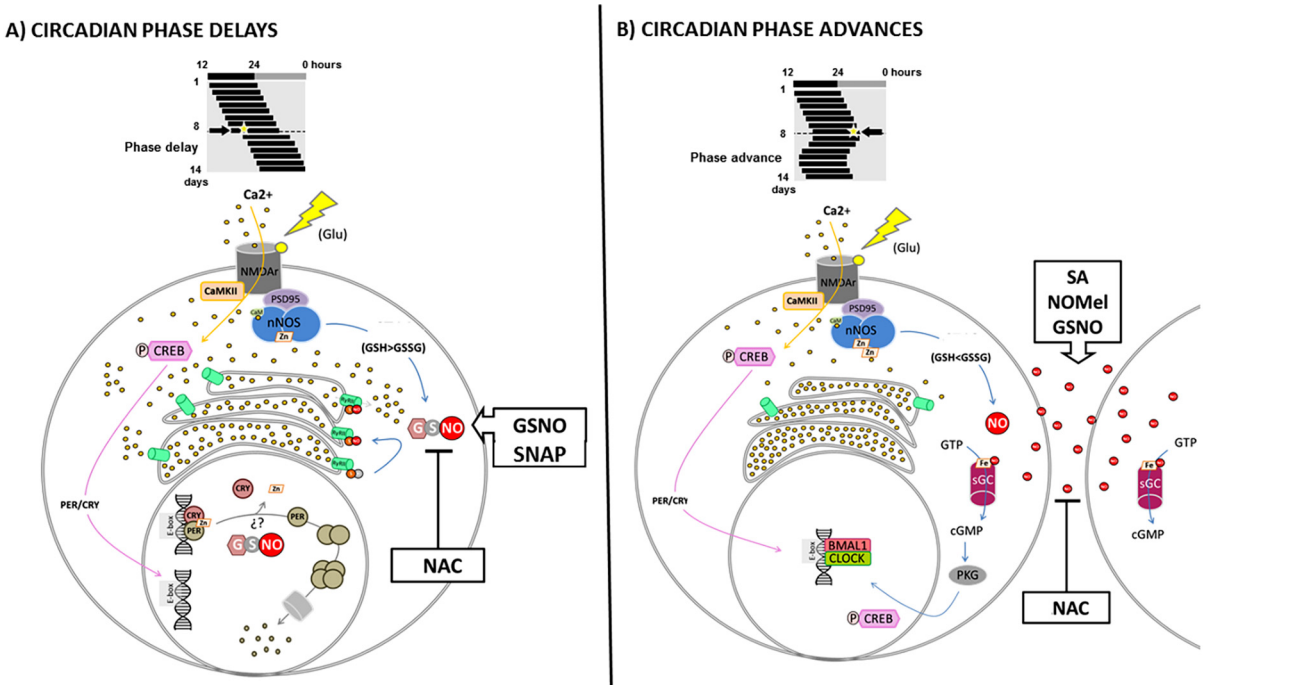

Fig. 2. Potential pathways for the action of NO· donors at the circadian clock. The left panel represents the signal transduction pathway for phase delays, while the right panel depicts the mechanisms leading to circadian phase advances. In both pathways, photic stimulation increases glutamatergic neurotransmission in suprachiasmatic neurons, and the open probability of N-methyl-D-aspartate receptor (NMDAr), rising $[Ca^{2+}]_i$ levels. A further interaction promotes Ca^{2+}-calmodulin kinase II (CaMKII) activity to phosphorylate calmodulin, which in turn activates nitric oxide synthase (nNOS) and NO· synthesis. The pathway for phase delays involves the aperture of ryanodine receptor (RyR), increasing $[Ca^{2+}]_i$. NO· heme-coordination of soluble guanylate cyclase (sGC), necessary for phase advances, increases synthesis of guanosine 3′,5′-cyclic monophosphate (cGMP), activating cGMP-dependent protein kinase (PKG). NO· is not a necessary messenger for photic-induced phase delays, while it is an intracellular and extracellular photic messenger at late subjective night. Drugs that release NO·, but also exhibit thiol reactivity generating S-nitroso groups, are effective to potentiate both pathways. This is the case for SNAP and GSNO, which potentially can generate S-nitrosation of RyR increasing its open probability. NO· donors having less ability for trans-S-nitrosation reaction, such as NOMel, and SA (also an HNO donor), only interact in the pathway for phase advances, in which NO· extracellular communication is essential. Redox gating (estimated by GSH/GSSG ratio) also generates different photic nitrergic messengers, such as GSNO at early subjective night for delays, or NO· for advances. GSNO could act also as an endogenous modulator of clock protein heterodimers by trans-S-nitrosation (PER2:CRY1) leading to PER2 aggregation/degradation. Modifications of the redox environment with NAC (or GSH) could impede nitrergic transmission by depletion of S-nitroso compounds, and/or by intracellular NO· scavenging, thus blocking photic phase shifts.

night, through Ca^{2+}/CaM signaling [93]. Also, melatonin induces the expression of genes of the antioxidant enzymes cupper zinc/manganese superoxide dismutase (SOD1/SOD2), glutathione peroxidase/reductase, and catalase in the hippocampus, brain cortex and cerebellum [92].

7.2. Redox/nitrergic gating of the circadian pathway

At the crossroads of classical and non-classical transduction, several components of the photic circadian pathway can be modulated by redox/NO·-dependent messengers. Starting from their input, S-nitrosation of NMDA receptors by nNOS nitrosative derivatives decrease their open probability exerting negative feedback on glutamatergic transmission [94]. Oxidative conditions increase the open probability of the RyR sarcoplasmic channel, while reductive conditions do the opposite [49]. Thiol-based redox modulation of enzymatic activity is proposed for negative feedback in NO·/cGMP transduction, and/or to counteract ROS/RNS generation. Thiol oxidation of critical cysteines changes nNOS activity from oxidase to reductase, decreasing NO· levels and generating GSNO [15,16]. It also promotes sGC desensitization to NO· [52]. Membrane excitability of SCN neurons is also modulated by redox changes altering circadian clock input-output by putative post-translational changes connected to cellular metabolism [95].

In this context, we propose that the modulation of redox state at the SCN alters circadian photic transduction. First, we have found that the GSH/GSSG ratio decreases throughout the circadian night [77]. Intracerebroventricular administration of NAC and GSH decreased the magnitude of both light-induced advances and delays. NAC is an effective thiol-scavenger which can target S-nitrosation at both RyR [42,34] and sGC [52], reducing its activity. It is known that cysteine, a precursor of GSH synthesis [96], is a product of NAC metabolism [53]. Thus, another possibility relies on the ability of both drugs to act as scavengers of NO· (i.e., by GSH, GSNO formation) and its nitrosative derivatives. In addition, the administration of both drugs without the light pulse generates phase delays in the early night. Through increasing the GSH/GSSG ratio, both compounds can generate similar circadian effects by changing the global redox state in SCN cells. This could be interpreted as an effective change on a circadian redox oscillator, affecting the phase of the circadian clock [95,88].

7.3. Clock proteins as targets in redox signaling

We have described above that post-translational redox regulation of circadian proteins can affect their activity as transcription factors, which sets the phase of the clock and its synchronization to light [11]. The stability of PER:CRY and CLOCK:BMAL1 heterodimers (which is mainly regulated by phosphorylation-ubiquitination) must be considered a key factor setting the phase of the circadian clock. In addition, PER1/2/3, CLOCK (o NPAS2) and BMAL1 clock proteins contain a characteristic PAS domain having a zinc tetra-thiolate [97] which could act as a sensor for redox potential. There is evidence that BMAL1 can be S-nitrosated *in vitro* [98]. In this context, we put forward the idea that clock proteins could be targeted by S-nitrosations as a putative regulatory component of the redox-circadian pathway. We have obtained

Table 1
Major pharmacological properties of NO· donor drugs demonstrated *in vivo*. The main by-products are those generated during the first steps of metabolism, or by spontaneous decomposition (for NAC, secondary glutathione generation is also indicated). The non-enzymatic pathway indicates that the donor drug reacts with an endogenous compound (e.g., S-nitrosothiol, ascorbate, metal ions) as an intermediate step to NO· formation. Thiol reactivity indicates the ability to form S-nitroso groups by transfer of NO· to thiols (i.e., trans-S-nitrosation). Drug targeting of RyR (canonic isoform 2 in cardiovascular tissue) involves an S-nitrosothiol formation at cysteine residues, while that of sGC, a NO·-heme coordination (secondary targets are not indicated for simplicity). #Oxidable cysteine residue. *Deplete S-nitrosations. Abbreviations: GSNOR, S-nitrosoglutathione reductase; mtADH, mitochondrial aldehyde dehydrogenase; GST, glutathione S-transferase; cytP450, cytochrome P450 complex; TRPV1, transient receptor potential cation channel 1. n.d.: not determined.

	main by-products	NO generation		thiol reactivity	Cardiovascular targets	Circadian targets	Circadian photic-effects
		enzymatic	non-enzymatic				
N-nitrosomelatonin (NOMel)	melatonin, NO·	n.d.	spontaneous, thiols	low	n.d.	sGC	phase advance
S-nitroso acetyl penicillamine (SNAP)	N-acetyl-D,L-penicillamine disulfide, NO·	n.d.	thiols	high	RyR2, sGC	RyR, sGC	phase delay/ advance
S-nitrosoglutation (GSNO)	glutathione, NO·	GSNOR	thiols, metal ions	high	RyR2, sGC	RyR, sGC	phase delay/ advance
S-nitrosocysteine (CysNO)	cysteine, NO·	n.d.	n.d.	high	R-SH#	R-SH#, PER2, BMAL1	n.d.
Sodium nitroprusside (SNP)	cyanide, NO·	n.d.	thiols, reductants, oxyhemoglobin	high	sGC	n.d.	n.d.
Glyceryl trinitrate (GTN)	1,2, glyceryl dinitrate, NO·	mtADH, GST, cytP450	thiols	high	sGC	n.d.	n.d.
Angeli's salt	HNO, NO·	n.d.	spontaneous, thiolates	low	TRPV1, sGC	sGC	phase advance
N-acetylcysteine (NAC)	cysteine (glutathione)	Non-generating	Non-generating	high*	RyR2, sGC*	RyR, sGC*	blocking

evidence *in vitro* using HEK-293 that PER2, and likely CRY2, have detectable oxidable cysteines, and Per2 and BMAL1 show changes by S-nitrosation [77]. This type of modification reduced the stability of the quaternary structure of both PER2 and BMAL1, decreasing the half-life of their monomeric versions and increasing the appearance of homodimers and multimers. In addition, bioinformatic modelling suggests that packing of PER2 and BMAL1 homodimer is favored by disulphide intramolecular bounds between PAS and bHLH domains. S-nitrosation was proposed to be an intermediate oxidative state of thiols towards disulphide bound formation [54]. We propose that these oxidative changes in the pool of clock proteins could be part of the mechanism leading to the stability, degradation, and/or inactivation of functional homo and heterodimers. Increasing NO· levels by light-activation of nNOS could destabilize the PER2:CRY1 heterodimer by zinc tetra-thiolate oxidation [99], allowing further PER2 homodimerization, multimerization, and degradation. In addition, the monomer/homodimer ratio could establish the degradation kinetic of clock proteins changing the phase of the circadian clock. Photic degradation of BMAL1 could be of importance when competing with CLOCK for the CLOCK:-BMAL1 [100] and/or NPAS2:BMAL1 [89] transcriptional activator complexes, although its role in the photic pathway is not clear as that of PER1-2. Despite in our model homodimerization of PER2 and BMAL1 could be mediated by the well-documented bHLH and PAS-domain interactions [100], to date there is no evidence for a role of ROS/RNS regulating this interaction.

8. A framework for the action of NO· donors in the circadian clock

We have provided insights on the pharmacology of NO· donors and its potential pathways at the circadian SCN clock (Fig. 2). Compounds having similar ability to increase NO· levels under physiological conditions, such as NOMel, SNAP, GSNO, and AS (which is also an HNO donor), stimulate photic advances. NO·-heme sGC activation and extracellular NO· communication at the SCN seem to be implicated in this effect. Of these NO· donors, only those that are S-nitrosothiols, such as SNAP and GSNO, will likely stimulate the phase-delaying pathway through trans-S-nitrosation of RyR2, increasing its open probability and $[Ca^{2+}]_i$. Circadian changes in redox gating (i.e., increased GSH/GSSG ratio) are also able to modulate the effect of NO· donors affecting its bioavailability and/or biotransformation. This gating could be of importance taken the reductive-oxidative switch in nNOS activity. While NO· behaves as an intra- and extracellular messenger in the phase advancing pathway, GSNO could be a potential endogenous redox messenger in the pathway for circadian delays, a possibility that should be tested. Indeed, the slightly modified cysteine NAC could decrease the effects of light pulses through competitive binding at GSH-docking site of nNOS, reducing both GSNO and NO· levels. Removing S-nitrosation of RyR2 seems to be a possibility for the inhibition of phase delays. However, this is probably not the case for sGC since its S-nitrosation may exert negative feedback for NO·-heme coordination, thus potentiation of light-induced phase advances should be expected through this mechanism.

Activity of *per1-2* genes at the mammalian SCN set the phase of the clock, with a peak at the end of the subjective day, decreasing throughout the circadian night. Light re-setting involves the induction of *per1-2* through binding of transcription factors (i.e., phosphorylated CREB) at the promoter region (E-box) (Fig. 2). Light-related redox regulation amay include liberation of PER2:CRY1 through GSNO trans-S-nitrosation at a zinc-finger tetra-thiolate domain (which helps to stabilize the heterodimer). Oxidizing conditions may also generate PER2 homodimerization/aggregation/degradation to contribute with this mechanism. During the late night, advancing the phase of the clock needs an advanced transcriptional activation of *per1-2* and extracellular NO· acting in neighboring SCN cells. For this not only the *de novo* synthesis of PER is important, but also accelerating its degradation, where S-nitrosation of PER2 generating homodimers and aggregates could play an important role.

9. Future directions

In this commentary we have presented evidence regarding the differential effects of the diverse NO· donors that are used for biochemical research. The pharmacological pathways and expected effects of NO· donors for clinical and experimental use in cardiovascular alterations are well known. To further sustain our argument, we have exemplified the heterogeneous activity of NO· donors by presenting the clearly distinct effects of such compounds on light-induced phase-shifts of mammalian circadian rhythms. While nNOS activation appears to be involved in both pathways for photic delays or advances of the clock, the mechanism bifurcates in the early or late subjective night (see Fig. 1). In the light of the observed changes, and the different NO· donor abilities, we propose that non-classic nitrergic transduction based

on RyR2 S-nitrosation, is operating phase delays at early night, while classic NO/cGMP/PKG pathway is needed for advances. Pathways can be also defined depending on the expected type and levels of nitrergic messenger (e.g., NO·, GSNO), and the available targets.

Table 1 summarizes the main properties of typical donors and its expected physiological effects at the cardiovascular and circadian systems. The dual capacity of S-nitrosothiols to increase NO· levels, as well as S-nitroso compounds, could be of importance when studying the effects of donors on the photic transduction pathway. A general rule is to consider two major class of donors: 1) those that increase NO· levels without major reactivity to thiols, such as NOMel and SA; 2) those having both effects, dividing them into two sub-classes: S-nitrosothiols, with the ability to trans-S-nitrosate cysteine thiols (SNAP, GSNO, CysNO), and nucleophiles with affinity for thiolates (SA). Most NO· donors that have thiol reactivity are taken to increase NO· levels through a previous S-nitrosothiol formation, which in turn will behave as the organic moiety for NO· liberation (see Table 1). For circadian entrainment, a tonic, sustained NO· transmission could be proposed for the phase-delaying pathway, thus involving NO· storage (i.e., GSNO favored at early night) and prolonged half-life, while acute NO· levels could activate the classic NO/cGMP/PKG pathway involved in phase advances. Of course, the redox environment can gate the effects of NO· donors favoring differential reactivity with targets, as well as half-life and bioavailability of messengers.

In summary, while nitrergic pathways play a fundamental role in diverse physiological mechanisms, biochemical and pharmacological research should carefully consider which is the NO· donor most suited to the experimental aims, since its chemical nature will certainly affect the outcome. Just saying “NO” is too simplistic to investigate this complex pathway of events leading to gene or ion channel activity and, in addition, the ever-changing cellular environment is also a variable that might affect the results of administering the same kind of compound.

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