VIP Nitrite Reduction Very Important Paper

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Generation of HNO and HSNO from Nitrite by Heme-Iron-Catalyzed Metabolism with H_2S**

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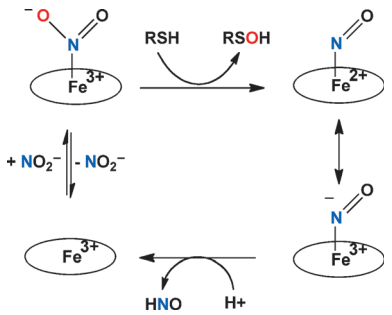
Nitrite has been shown in the past decade to be an important source of nitric oxide that acts as a vasodilator and intrinsic signaling molecule.[1] Numerous studies have proved that nitrite can be reduced in vivo, either non-enzymatically[2a,b] or enzymatically, in reactions catalyzed by xanthine oxidase,[2c] deoxyhaemoglobin,[2d] deoxymyoglobin,[2e] cytochrome c,[2f] or by thiol and metal-center-assisted processes inside the cell.[2g] The mechanism of the last process has recently been studied in detail, and has demonstrated that thiols (cysteine and gluthathione) stimulate water-soluble Fe^{3+}-porphyrins to have nitrite reductase activity through an oxygen atom transfer (OAT) mechanism (Scheme 1) that leads to increased



Scheme 1. OAT mechanism.[3]

formation of Fe^{2+}-NO-porphyrin and the corresponding sulfenic acid.[3a] Furthermore, the same research group showed recently that in fact the main products of this reaction are NO and its reduced congener, nitroxyl (HNO).[3b]

However, the smallest thiol that can diffuse without any constrains[4a] and which could reach the active sites of most of the metalloproteins, that is, hydrogen sulfide, has not yet been considered as a possible regulator of this reaction. Nonetheless, in clinical praxis, nitrite is used as an antidote for hydrogen sulfide (H_2S) poisoning, being even superior to oxygen.[1d] The mechanism by which nitrite acts in such severe cases has remained elusive.

Together with its physiological roles, beneficial effects of H_2S on various animal disease models have been documented and they, in part, resemble those of nitrite.[5] The cross-talk of the H_2S and NO signaling pathways has only recently been recognized as a possible explanation for various beneficial effects of H_2S in our bodies.[6]

We have demonstrated recently that H_2S and acidified nitrite could form thionitrous acid (HSNO),[6a] but this reaction is too slow to occur at a physiological pH value.[7] Indeed, an increasing nitrite concentration in a buffered sodium sulfide solution did not affect the spontaneous decay of H_2S from the solution, as measured with an H_2S electrode (not shown). However, when we measured the consumption of H_2S (100 μM) by Jurkat cells (2.5×10^5 cell mL^{-1}) in the absence and presence of 100 μM nitrite (see Figure S1A in the Supporting Information), H_2S disappeared from the medium faster than in the control, an effect that was even more pronounced when a higher concentration of nitrite was used (1 mM). H_2S consumption was completely abrogated following cell inactivation by heat (60 °C for 1 h) or pretreatment with paraformaldehyde, and markedly inhibited by cyanide. These results suggest that nitrite and sulfide could react intracellularly through the involvement of a metal-based metabolic pathway (see Figure S1B in the Supporting Information).

We next measured the formation of NO in human umbilical vein endothelial cells (HUVECs) loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) and treated with 100 μM H_2S, 100 μM nitrite, or a combination of the two (see Figure S2 in the Supporting Information). Incubation with nitrite caused a slight increase in the intracellular NO formed, in agreement with the previous observation of nitrite processing in the cell.[2g] However, the combination of nitrite and H_2S gave the strongest signal by far, thus implying that these two molecules induce the generation of NO inside cells.

Furthermore, we attempted to measure the intracellular generation of HNO. We used a recently developed fluorescence sensor (CuBOT1) for the intracellular detection of nitroxyl.[8,6a] The cells were loaded with 5 μM CuBOT1 for 20 min and then exposed to 100 μM nitrite, sulfide, or a combination of the two for 30 min. The data obtained (see Figure S3 in the Supporting Information) clearly demonstrated the formation of HNO when the combination of nitrite and sulfide was used, as evident by the strong increase in the fluorescence of intracellular CuBOT1 (ca. eightfold, $n=20$ cells). Individual treatment with nitrite and hydrogen

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sulfide did not induce any significant change, in line with previous reports.

If metal centers play a role in the H_2S-facilitated nitrite-reductase activity, then the majority of the HNO will be generated in mitochondria, as it is the organelle that is richest in heme-iron centers. To test this, cells treated with nitrite and hydrogen sulfide were incubated with both CuBOT1 and Mito Tracker Red, and the localization of the fluorescence compared.

The results in Figure 1 clearly demonstrate that the majority of the HNO-induced fluorescence is localized in mitochondria. No observable change in the basal fluorescence

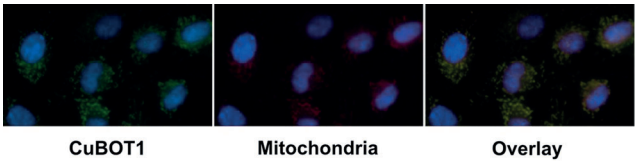


Figure 1. Mitochondrial localization of HNO formed from nitrite and sulfide. HUVECs treated with 100 μM nitrite and 100 μM sulfide were stained with a fluorescence sensor for nitroxyl (CuBOT1, green) and a fluorescence sensor for mitochondria (Mito Tracker Red, red).

could be found in Rho^0 HUVE cells depleted of mitochondria[9] (see Figure S4 in the Supporting Information). Furthermore, when left-ventricular cardiomyocytes (cells rich in mitochondria) were aerobically incubated with nitrite, sulfide, or both and analyzed by EPR spectroscopy at 80 K, a strong EPR signal of the heme Fe^{2+}-nitrosyl moiety[10] was observed when nitrite and sulfide were added, similar to that observed with nitrite alone under anaerobic conditions (see Figure S5 in the Supporting Information). All these data pointed towards the conclusion that the nitrite-/sulfide-induced formation of NO and HNO takes place through a heme-iron-catalyzed pathway within mitochondria, and suggests that H_2S may be the elusive thiol responsible for the reduction of nitrite.

To understand these cellular experiments on a molecular level we used a water-soluble porphyrin [Fe^{3+}(P)] with eight negative charges on a ligand periphery (see Figure S6 in the Supporting Information).[11] Recent studies demonstrated that the reaction of a similar porphyrin with thiols and nitrite is in fact catalytic, and follows the simplified reaction pathway shown in Scheme 1.[3b] Indeed, when the reaction was followed electrochemically, the addition of 30 μM [Fe^{3+}(P)] caused the complete removal of 200 μM H_2S in the presence of 1 mM nitrite (see Figure S7 in the Supporting Information).

Three-syringe stopped-flow kinetic measurements and global spectral analysis were performed with 10 μM [Fe^{3+}(P)] and various concentrations of Na_2S and $NaNO_2$. The reaction was carried out at a physiological pH value (7.4) but under anaerobic conditions, since a strong bleaching and precipitation of sulfur was observed when the reaction was performed aerobically (not shown).

Nitrite itself did not induce any spectral change over a period of a few hours, consistent with previous observations.[3] Unlike cysteine and glutathione,[3a] H_2S caused the fast reduction of [Fe^{3+}(P)] ($k_{red} = 9.5 \pm 0.5\,M^{-1}s^{-1}$), with a characteristic shift of the Soret band from 398 nm to 432 nm (see Figure S8 A in the Supporting Information). However, when mixed together, the formation of [Fe^{2+}(P)(NO)] was observed with a characteristic Soret band at 416 nm and Q band at 556 nm (Figure 2). Global spectral analysis of the correspond-

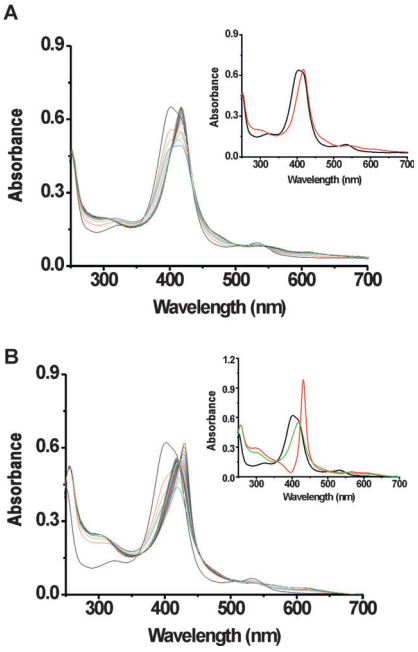


Figure 2. Time-resolved UV/Vis spectra of the reaction mixture (in 300 mM phosphate buffer pH 7.4) containing [Fe^{3+}(P)], Na_2S, and nitrite. A) 10 μM [Fe^{3+}(P)] was mixed with 5 mM nitrite and 1 mM Na_2S (monitored over 647 s; integration time 2 ms). Inset: predicted spectra of formed species (black: starting species, red: product species). B) 10 μM [Fe^{3+}(P)] was mixed with 1 mM nitrite and 5 mM Na_2S (monitored over 373 s; integration time 2 ms). Inset: predicted spectra of formed species (black: starting species, red: intermediate species, green: product species).

ing time-resolved spectra revealed only a single-step process when nitrite was present in excess over sulfide (Figure 2 A, and see Figure S8B,C in the Supporting Information): [Fe^{3+}(P)] was transformed to [Fe^{2+}(P)(NO)], thus suggesting the operation of an OAT mechanism (Scheme 1). Our attempts to trap the corresponding sulfenic acid (HSOH) with dimedone were, however, unsuccessful, but this is expected as HSOH is extremely unstable. Slow precipitation of sulfur was, however, indicative of the oxidation of sulfide.

Conversely, a different reaction mechanism was observed when sulfide was in excess over nitrite. Spectral analysis clearly revealed the existence of the two steps: 1) the reduction of [Fe^{3+}(P)] to [Fe^{2+}(P)] (shift in the Soret band from 398 nm to 432 nm) and 2) formation of [Fe^{2+}(P)(NO)] (shift in the Soret band from 432 nm to 416 nm). The involvement of an intermediate [Fe^{2+}(P)] becomes more pronounced as excess of sulfide is increased from 1:2 (see Figure S8D,E in the Supporting Information) to 1:5 nitrite/sulfide (Figure 2 B).

EPR analysis of the corresponding frozen reaction mixtures confirmed the UV/Vis observation. Namely, the high-spin spectrum of [Fe^{3+}(P)] with $g = 5.603$ was not significantly affected by 1 mM nitrite (see Figure S9 in the Supporting Information). The addition of sulfide to [Fe^{3+}(P)] led to the loss of the high-spin signal and the appearance of a low-spin Fe^{3+} complex with $g_x = 2.463$, $g_y = 2.249$, and $g_z = 1.902$. An additional peak was observable at $g = 1.997$, which we assigned to HS$^{\bullet}$. Such EPR spectra suggest that the observed intermediate is an [Fe^{3+}(P)(HS^-)] species (EPR active, low-spin species, as in the case of heme Fe^{3+}-(H)SH adducts),[12] which decomposes to HS$^{\bullet}$ (EPR active) and [Fe^{2+}(P)] (EPR silent), as summarized in Equations (1) and (2).

$$[Fe^{3+}(P)] + HS^- \rightleftharpoons [Fe^{3+}(P)(HS^-)] \quad (1)$$

$$[Fe^{3+}(P)(HS^-)] \rightarrow [Fe^{2+}(P)] + HS^{\bullet} \quad (2)$$

The combination of 1 mM nitrite and 10 mM sulfide leads to the formation of a mixture of low-spin [Fe^{3+}(P)(HS^-)] and [Fe^{2+}(P)(NO)] (see Figure S9 in the Supporting Information). This is in agreement with the UV/Vis observations, thus confirming the formation of the intermediate [Fe^{2+}(P)] followed by its nitrite reductase activity [Eq. (3)]. The Fe^{3+}-

$$\begin{aligned}&[Fe^{2+}(P)] + NO_2^- + 2H^+ \rightarrow [Fe^{3+}(P)(NO)] + H_2O\\ &[Fe^{3+}(P)(NO)] \leftrightarrow [Fe^{2+}(P)(NO^+)]\end{aligned} \quad (3)$$

nitrosyl complex (with a Soret band at 424 nm; see Figure S10A in the Supporting Information) formed through Equation (3) is known to undergo spontaneous or nitrite-catalyzed reductive nitrosylation to give the final [Fe^{2+}(P)(NO)].[13a–d] A proposed mechanism involves a direct nucleophilic attack of H_2O/OH^- ($k = 2.4 \times 10^{-4}\ s^{-1}$) or NO_2^- ($k = 2.1\ M^{-1}s^{-1}$), respectively, as a rate-determining step.[13a]

However, starting from [Fe^{3+}(P)] (Figure 3) we could not clearly observe an intermediate [Fe^{3+}(P)(NO)], which is consistent with it being quickly scavenged by another nucleophile, HS^- in our case. Indeed, when [Fe^{3+}(P)(NO)] was mixed with sulfide, it immediately gave [Fe^{2+}(P)(NO)] (see Figure S10A in the Supporting Information). A closer look at the spectral changes obtained upon addition of nitrite to a mixture of [Fe^{2+}(P)] and sulfide revealed an additional species on the way to [Fe^{2+}(P)(NO)] (see Figure S10B in the Supporting Information).

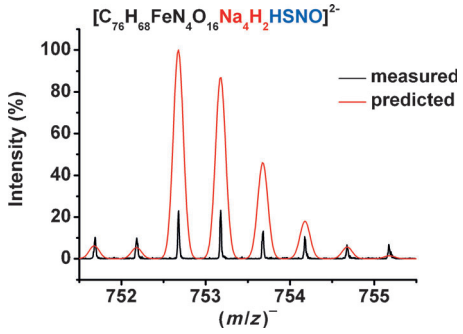


Figure 3. Cryospray ESI-TOF MS detection of HSNO coordinated to Fe^{2+} porphyrin. Red: predicted isotopic distribution; black: measured isotopic distribution.

The [Fe^{3+}(P)(NO)] complex has [Fe^{2+}(P)(NO^+)] character [Eq. (3)][11] and is prone to nucleophilic attack to form [Fe^{2+}(P)(NO-Nu)] adducts.[13a–c] Sulfide is a much better nucleophile than nitrite, which explains its faster reaction. The initial product of the sulfide attack would be coordinated thionitrous acid [HSNO; Eq. (4)].

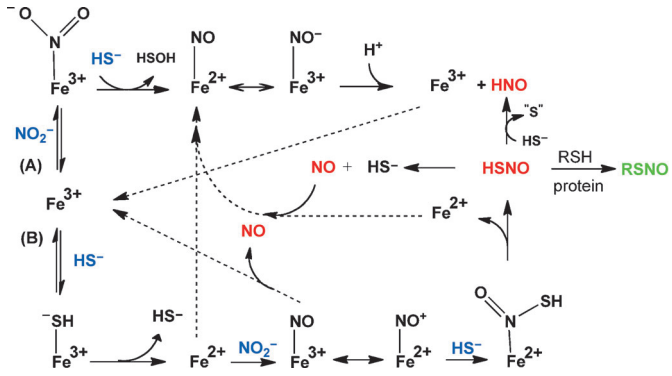
$$[Fe^{2+}(P)(NO^+)] + HS^- \rightarrow [Fe^{2+}(P)(HSNO)] \quad (4)$$

To understand whether this reaction intermediate is indeed formed we used high-resolution cryospray ESI-TOF mass spectrometry in a negative mode, which allowed us to spray the reaction mixture at −20 °C and thus preserve unstable species that would otherwise be destroyed by standard high-temperature ionization. When we sprayed the reaction mixture of [Fe^{3+}(P)(NO)] and H_2S we were able to detect HSNO coordinated to Fe^{2+}, as confirmed by the high mass accuracy and predicted isotope distribution (Figure 3; see also Figure S11 in the Supporting Information). This observation suggests that the reaction shown in Equation (4) indeed takes place.

We also observed a doubly charged signal at m/z 726.6852 that corresponds to the Fe^{3+}-HS^- adduct (calculated m/z 726.6860), as well as the doubly charged signals at m/z 676.7186 and m/z 699.2005, which correspond to [Fe^{3+}(P)] and [Fe^{2+}(P)], respectively (see Figure S11 in the Supporting Information). This is in accord with the composition of the reaction mixture resulting from Equations (1) and (2) and the corresponding EPR spectra (see Figure S9 in the Supporting Information). Thus, based on both the EPR and mass spectra we were able to identify a very rare case of sulfide bound to synthetic ferric porphyrin,[14a,b] which has not yet been observed in the aqueous solution. This may be a consequence of a specific, highly negatively charged metal environment that is less favorable for iron reduction and hydrogen-bonding interactions with HS^-.[14c]

We[6b] and others[6c] have recently observed an Fe-HSNO intermediate in the reaction of nitroprusside with H_2S.[6b,c] HSNO could either decompose homolytically to give NO (resulting in [Fe^{2+}(P)(NO)]↔[Fe^{3+}(P)(NO^-)]) and HS$^{\bullet}$, or it can react further with H_2S to give HNO.[6a] Both reaction pathways would eventually give the HNO observed in our cellular studies (Figure 1, see also Figure S3 in the Supporting Information). Released HNO could dimerize to give N_2O.[15] We also measured the generation of N_2O in our model system in which 1 mM nitrite and 10 mM H_2S were mixed with 1 mM pophyrin. N_2O was measured by GC-MS and the authenticity of the signal confirmed using ^{15}N-labeled nitrite. Selected ion monitoring to either m/z 44 or m/z 46 gave a clear signal corresponding to nitrous oxide that increased over the course of the reaction (see Figure S12 in the Supporting Information).

On the basis of all these data, an overall reaction scheme with two pathways (A and B) is proposed (Scheme 2). Both paths could explain the generation of HNO in cells, but under



Scheme 2. Proposed reaction mechanism for the H_2S-assisted heme-catalyzed reduction of nitrite, starting from [Fe^{3+}(P)].

physiological conditions the excess of H_2S over nitrite would be the more common situation, thus meaning that intermediate formation of HSNO is probable (path B). Since a general mechanism of reductive nitrosylation involves a dissociation of the Fe^{2+}-(NO-Nu) adduct,[13a] this will be a source of HSNO in our case. It potentially leads to further transnitrosation through the cell, or locally as observed previously.[2g] To test this hypothesis we used the reaction mixture containing the Fe^{3+}-porphyrin with nitrite alone, nitrite and sulfide in combination, as well as the Fe^{3+}-nitrosyl complex and added bovin serum albumin. The protein content was analyzed for its *S*-nitrosothiol content after 15 min of incubation (see Figure S13 in the Supporting Information), and clearly showed that only the combination of porphyrin, nitrite, and sulfide catalytically generates a sufficient amount of HSNO, a potent agent for protein *S*-nitrosation.

Taken together, these data give an insight into the possible mechanism for intracellular nitrite reduction and its subsequent physiological effects. They also offer a possible explanation for the antidote effect of nitrite in H_2S poisoning: generation of NO, HNO, and HSNO could lead to an increase in the blood flow, and finally, better oxygen delivery to the tissue.[6a,16] Additionally, these results demonstrate that H_2S can transform biological metal-nitrosyl sources into *S*-nitrosothiols via HSNO and offers another mechanism for the physiological generation of HNO. It remains to be elucidated whether H_2S is indeed the elusive thiol responsible for the intracellular reduction of nitrite, its beneficial pharmacological effects, and what would be the downstream effects of this reaction on cellular metabolism.

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