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Forum Article

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Discussing Endogenous NO•/HNO Interconversion Aided by Phenolic Drugs and Vitamins

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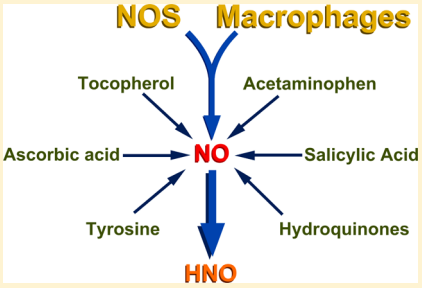
Supporting Information

ABSTRACT: The reduction of NO• to HNO/NO⁻ under biologically compatible conditions has always been thought as unlikely, mostly because of the negative reduction potential: $E°(NO^{\bullet},H^{+}/HNO) = -0.55$ V vs NHE at physiological pH. Nonetheless, during the past decade, several works hinted at the possible NO-to-HNO conversion mediated by moderate biological reductants. Very recently, we have shown that the reaction of NO• with ascorbate and aromatic alcohols occurs through a proton-coupled nucleophilic attack (PCNA) of the alcohol to NO•, yielding an intermediate RO−N(H)O• species, which further decomposes to release HNO. For the present work, we decided to inspect whether other common biological aromatic alcohols obtained from foods, such as Vitamin E, or used as over-the-counter drugs, like aspirin, are able to undergo the reaction. The positive results suggest that the conversion of NO to HNO could occur far more commonly than previously expected. Taking these as the starting point, we set to review our and other groups' previous reports on the possible NO-to-HNO conversion mediated by biological compounds including phenolic drugs and vitamins, as well as several thiol-bearing compounds. Analysis of revised data prompted us to ask ourselves the following key questions: What are the most likely physio/pathological conditions for NO•-to-HNO conversion to take place? Which effects usually attributed to NO• are indeed mediated by HNO? These inquiries are discussed in the context of 2 decades of NO and HNO research.



1. BACKGROUND

The reduction of NO• to HNO/NO⁻ in aqueous solutions has generally been disregarded as a significant process in biologically compatible conditions because of the negative reduction potential of NO• [$E°(NO^{\bullet}/NO^{-}) = -0.81$ V vs NHE].[1,2] However, at physiological pH, the reduction of NO• to the protonated HNO has a more favorable redox potential, being $E°(NO^{\bullet},H^{+}/HNO) = -0.11$ V vs NHE and $E°' \approx -0.5$ V at pH 7.[2] It is still hard to imagine which biological reductants could afford it because ubiquitous compounds such as NADH and cysteine have reduction potentials around −0.3 V at biological pH. Therefore, one-electron reduction of NO• to HNO has not received much attention. It is important to mention that the above-mentioned reduction potential is the result of an estimation because it cannot be measured directly as a result of the irreversibility of the NO•/HNO redox couple. This assessment was done by Shafirovich and Lymar about 13 years ago,[2] assuming $\Delta G_f°[HNO(aq)] \sim 115$ kJ/mol. However, it is important to note that if this value is overestimated by 20−30 kJ/mol, the NO•/HNO redox couple would be much closer to the thermodynamic range of many biological reductants.

Nonetheless, the reactions of NO•(g) with alcohols (ROH) and hydroperoxides (ROOH) in oxidized polyolefin films have been studied in the past, resulting in the formation of alkylnitrites (RONO) or alkylnitrates ($RONO_2$), which was evidenced by Fourier transform infrared.[3] The authors even proposed HNO as a possible intermediate, claiming as evidence

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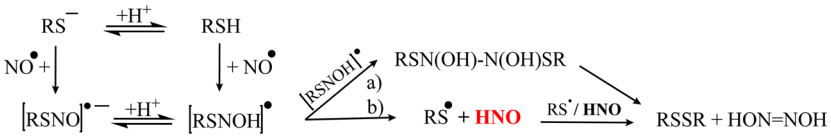
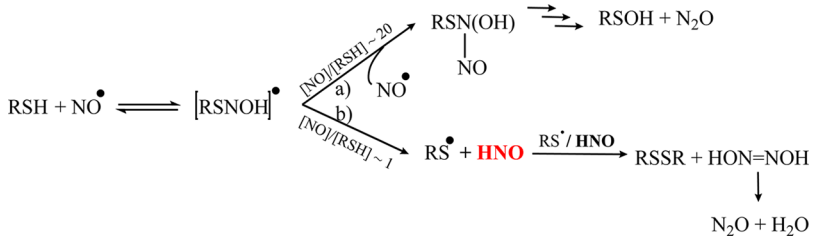
Scheme 1. Possible Mechanisms for the Reaction of NO with Thiols



Scheme 2. Possible Mechanisms for the Reaction of NO with Thiols



detection of a pale-yellow-green gas.[4,5] Also, some reports show that alcohol-derived "alkoxyl" radicals R−$CH_2O^•$ can react with NO• to yield HNO through a disproportionation reaction, which also produces the corresponding ketone. Moreover, other studies showed that nitric oxide can react with phenols to form phenoxyl radicals, as evidenced by electron paramagnetic resonance.[6] Similar results were obtained with 3,4-dihydroxyphenylacetic acid (DOPAC), which yielded the corresponding semiquinone radical anion.[9] Both facts suggest HNO formation, which in the second case was indirectly revealed by conversion of metmyoglobin to nitrosylmyoglobin. Finally, Poderoso et al.[7] showed that truncated forms of ubiquinol react with NO• to form HNO,[8] which was indirectly detected by metmyoglobin trapping and N_2O formation.

Besides alcohols, the reactions of NO and NO donors with thiols have also been studied. In 1982, Pryor and co-workers found[9] that aliphatic and aromatic thiols react with NO, producing N_2O and the corresponding disulfide under anaerobic conditions. The initial proposed step involves the addition of NO• to the thiol, which forms an intermediate RS−NO• (Scheme 1) in equilibrium with its protonated form. The authors proposed dimerization of the intermediate and ulterior decomposition to disulfide and hyponitrous acid, a precursor of N_2O (pathway a). Although dimerization is not impossible, it is not expected to be favorable because of the high reactivity and low concentration of the radical intermediate. An alternative mechanism could involve decomposition of the [RSN−OH]• intermediate to produce HNO and RS•, which would rapidly dimerize to produce hyponitrous acid and the observed disulfide (pathway b).

More than 10 years later, DeMaster and co-workers[10] studied the anaerobic reaction of albumin (a protein containing −SH groups) and other thiols such as glutathione with NO• or DEA-NO. These reactions, which took place in minutes, were shown to produce also N_2O but the corresponding sulfenic acid RS−OH instead of disulfides. It should be noted that in this case NO• was in a large excess, ca. 10−20 times with respect to the thiols, and the concentrations of thiols were in the nanomolar range, several orders of magnitude more diluted than that in the experiments conducted by Pryor. The mechanism proposed by the authors (Scheme 2) involved, again, the initial formation of [RSN−OH]•, which produced an *S*-(*N*-nitroso)hydroxylamino intermediate by reaction with a second molecule of NO• (pathway a). Decomposition of the [RSN−OH]• intermediate (pathway b) was not observed, probably because of the high NO•/RSH ratio, which definitely favors the addition of a second NO• molecule in pathway a.

Finally, other relevant studies were carried out with NO• donors. Kirsch and co-workers[11] studied the interplay between ascorbic acid and *N*-acetyl-*N*-nitrosotryptophan (NANT) in aerobic and anaerobic conditions. They found that ascorbic acid promotes the release of NO• from NANT to give N-acetylated tryptophan and an ascorbyl radical. Authors observed N_2O as a product, suggesting the presence of HNO. The proposed mechanism involved the formation of *O*-nitrosoascorbate, which could disproportionate into dehydroascorbic acid (DHA) and HNO. In another work by Kirsch and co-workers,[12] the reaction between *S*-nitrosothiols and ascorbate was studied, and HNO formation was suggested, once more, in a mechanism involving *O*-nitrosoascorbate, which decays to HNO and DHA.

Although all of the above-mentioned studies suggested, and indirectly showed, azanone production upon NO• reaction with alcohols (or thiols), most of them did not prove its presence unequivocally, and the underlying reaction mechanisms were not thoroughly analyzed. Recently, we suggested that the reactions of NO• with ascorbate and aromatic alcohols could be mediated by a proton-coupled nucleophilic attack (PCNA) of the alcohol or alkoxide to NO•, yielding an intermediate RO−N(H)O• species, similar to the [RS−NOH]• intermediate proposed in the case of thiols. This intermediate, which was observed by cryogenic mass spectrometry, further decomposes to release HNO.[13] As directly evidenced using two different selective HNO trapping and sensing devices, these results (as will be discussed in the present review) could have wide biological implications given the ubiquitous presence of ascorbate, as well as of several aromatic alcohols such as tyrosine, and its coexistence with NO•, in natural systems.

Particularly relevant in light of these reactions are clinical and pharmacological studies involving phenolic drugs and NO• (or precursors), such as aspirin and L-arginine. Aspirin has a phenolic group protected as an acetyl ester, which gets deprotected mainly in the liver. An improvement in myocardial recovery was observed after ischemia when both aspirin and L-arginine were administered simultaneously,[14] an observed clinical effect that is typical of HNO donors.[15] In a different context, the acetaminophen-induced hepatotoxicity in the presence of NO• has recently been studied.[16−19] Acetaminophen is a mild analgesic and antipyretic agent known to cause hepatic necrosis at toxic doses. Although, at first sight, this may

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be due to a direct interaction of reactive acetaminophen metabolites with hepatocyte proteins, new studies have suggested that NO• contributes to the pathophysiological process.[20] Moreover, apart from vitamin C, other biological relevant compounds such as vitamin B6 and vitamin E have −OH groups attached to aromatic rings and could lead to HNO from NO•. The interaction between vitamin E (tocopherol) and NO• has been studied.[21] Although the authors demonstrated that α-tocopherol is oxidized by NO• to an α-tocopheroxyl radical, a reaction that should result in the formation of HNO and finally N_2O, incubation of dilauroylphosphatidylcholine (DLPC) liposomes containing α-tocopherol with NO• resulted in no significant formation of N_2O. This could be due to interaction of the eventually produced HNO with the alkylammonium groups in the liposomes, which can work as oxidizing agents, or to steric hindrance of the −OH group by the liposomes. In summary, pharmacological data also suggest NO-to-HNO conversion mediated by phenolic drugs.

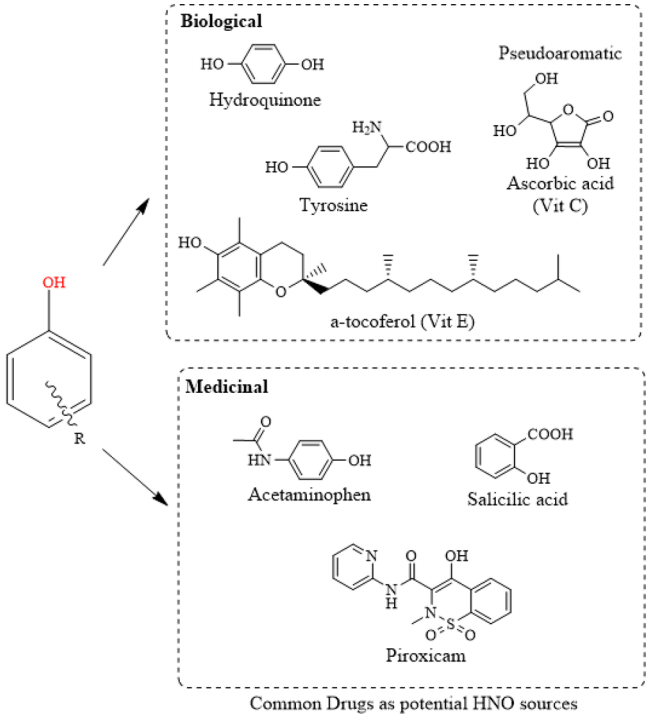
In the present work, we thus decided to, first, review the evidence supporting NO-to-HNO conversion by aromatic alcohols and expand it with the study of the NO reaction with common phenolic drugs and vitamins. Second, we will present a detailed mechanistic analysis including new density functional theory (DFT) calculations. Then, we will review the data showing that the reaction actually happens in a biological context. Finally, we will discuss the biological relevance of NO-to-HNO conversion in a broader context.

2. AROMATIC ALCOHOLS REACT WITH NO• TO PRODUCE HNO

A detailed study of how NO• reactions with electron-rich compounds are able to yield HNO through a PCNA was recently performed by our group using aromatic and "pseudoaromatic" alcohols—ascorbate anion ($AscH^-$), phenol (PhOH), hydroquinone (HQ), and tyrosine (Y)—as hydrogen/electron sources in aqueous solutions (see Scheme 3).[13] The key issue at hand was to actually provide sound evidence that HNO was produced by this seemingly unexpected mechanism, and thus we used several methods to identify its presence unambiguously. Our first approach was to measure the conversion of Mn^{III}TCPP [TCPP = tris(1-chloro-2-propyl)phosphate] to the corresponding nitrosyl complex Mn^{II}TCPP-NO, a typical {MnNO}^{6} adduct in the Enemark−Feltham notation,[22] by using UV−vis spectroscopy.[13,23] Resulting spectral changes obtained after mixing of an anaerobic NO• solution with ascorbate ($AscH^-$), which are characteristic of manganese(III) nitrosylation by HNO, have been observed.[13,24] Because this manganese(III) porphyrin reacts neither with free NO•[24] nor with ascorbate,[25] these results strongly suggested HNO production. Similar results were obtained with HQ, Y, and PhOH but not with aliphatic alcohols, such as methanol, D-mannitol, or malic acid.[13]

The second approach used to determine HNO production relied on the recently developed HNO-selective electrode, which allows for time-resolved quantification.[26−29] The corresponding amperometric signal (which is directly proportional to HNO concentration) versus time plot after the addition of either $AscH^-$, HQ, or Y to anaerobic aqueous solutions of NO• shows production of HNO. As expected for a bimolecular reaction, the HNO concentration[29] is linearly dependent on both $AscH^-$ and NO• concentrations.

Scheme 3. Aromatic and "Pseudoaromatic" Alcohols React with NO-Releasing HNO



Comparative kinetics for all (previous and present) HNO-producing reactions are shown in Table 1. The reported rate constant (k_{app}) corresponds to the apparent bimolecular rate constant for effective HNO production, as determined by the electrochemical method and as defined by the following equation:[13]

$$d[HNO]/dt = k_{app}[NO][R{-}OH]$$

The results show that k_{app} has a moderately narrow range of less than 2 orders of magnitude, from about 0.1 to 8 M^{-1} s^{-1}, and corresponds to a relatively slow reaction compared with NO• reactions with other small molecules or metallic centers. However, it should be borne in mind that the plasmatic concentration of ascorbate and antioxidant drugs is rather high, ca. 5−1000 times higher than the 2 μM concentration used in the experiments, as can be observed in Table 1. Therefore, the reaction is likely to happen nonetheless (see the section on biological significance for further analysis). Although the correlation is not perfect, those alcohols with smaller redox potentials (like ascorbate and hydroquinone) tend to show faster rates, while some with more positive values, like tyrosine, tend to have lower rates. This apparent trend, as will be shown later, can be explained in the context of the proposed reaction mechanism. The table also shows that, for all compounds, peak HNO concentrations for standardized NO• and ROH concentrations ([NO] = 0.2 mM and [ROH] = 2 μM), are in the nanomolar range.

3. VITAMIN E ALSO PRODUCES HNO FROM NO

By using the same methods as those described before,[13] we analyzed whether other vitamins (see Scheme 3 and Table 1) with phenolic groups, apart from vitamin C, could produce HNO by means of the above-described reaction. Figure SI1 shows the HNO-selective electrode signal versus time plot after

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Table 1. Comparative Kinetics of HNO-Producing Reactions

compound	$E^{\circ\prime}$(pH 7; RX•,H+/RXH) (V)	PEC[a] (μg/mL, μM)	k_{app} (M^{-1} s^{-1})	$[HNO]_{max}$ (nM)[b]	ref for $E^{\circ\prime}$	ref for PEC
ascorbate	0.28	8.8−440, 50−2500	8.0 ± 0.5	30	30	31
hydroquinone	0.10		6.1 ± 0.4	25	30	
phenol	0.91		3.2 ± 0.4	20	30	
tyrosine	0.97		0.9 ± 0.4	28	30	
tocopherol	0.48	129, 300	3.3 ± 0.4	47	32	33
salicylic acid	1.10	110−160, 800−1600	0.34 ± 0.06	4	34	35
acetaminophen	0.70	10−20, 70−130	1.4 ± 0.6	56	36	37
piroxicam	0.80	4.5, 10	0.26 ± 0.04	5	38	39

[a]Plasmatic effective concentration (PEC): concentration of a drug in plasma required to produce a desired pharmacological effect in most patients. [b]Calculated in all cases for the following standard conditions: [NO] = 0.2 mM and [ROH] = 2 μM.

repetitive additions of NO to a solution of α-tocopherol (a form of vitamin E that is preferentially absorbed and accumulated in humans).[40] The plot clearly shows how HNO is produced after each addition.

The impact of the incubation of phosphatidylcholine liposomes containing α-tocopherol with NO• was also studied.[20] It was observed that the presence of phospholipids alters the reactivity of the −OH groups of α-tocopherol. This could be due to interaction of the eventually produced HNO with the ammonium groups in the liposomes, which can work as oxidizing agents, or a steric hindrance to reach the −OH group produced by the presence of liposomes containing long alkyl chains. Further analysis is needed to address these issues, which are out of the scope of the present review.

4. COMMON DRUGS WITH −OH GROUPS AS POTENTIAL HNO SOURCES

Another source of circulating aromatic alcohols is commonly used drugs, or their primary metabolites (Scheme 3). As commented before, the plasmatic concentrations of these compounds are rather high, as shown in Table 1. Therefore, we decided also to test whether they can react with NO• to yield azanone. We started with the widely used aspirin, acetylsalicylic acid (ASA), an aromatic acetyl ester. ASA is a prodrug whose primary active metabolite is the aromatic alcohol salicylic acid (SA). ASA is transformed into SA in the stomach, in the intestinal mucosa, in the blood, and mainly in the liver.[41,42] Although SA is responsible for most antiinflammatory and analgesic effects, ASA is the active moiety for the antiplatelet-aggregating effect.[42] As expected, while the ester (ASA) is unable to react with NO• because of ester protection on the −OH group, HNO production is clearly observed with SA (Figure SI2A).

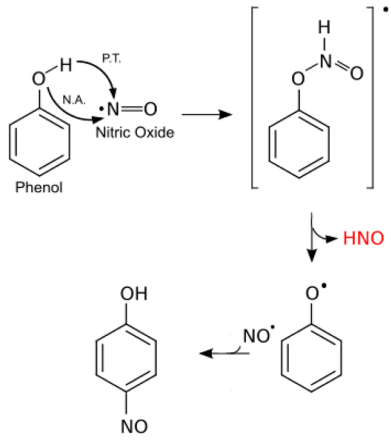
We also studied piroxicam as a reactant. Piroxicam is an extensively used nonsteroidal antiinflammatory drug and an antiarthritic drug, which acts as a nonselective cyclooxygenase (COX) inhibitor possessing both analgesic and antipyretic properties.[43,44] HNO generation was also observed after the addition of NO (Figure SI2A).

Acetaminophen [*N*-(4-hydroxyphenyl)acetamide, also known as paracetamol] is another commonly used over-the-counter analgesic and antipyretic bearing a phenol group.[43] As for SA, the current versus time plot for the addition of NO to a solution of paracetamol (Figure SI2B) clearly indicates significant HNO production. Interestingly, the signal for paracetamol is much more intense than that for the other drugs that had been studied. Also, the conversion of Mn^{III}TCPP to the corresponding nitrosyl complex Mn^{II}TCPP-NO by using UV−vis spectroscopy was observed (data not shown).

5. MECHANISTIC ANALYSIS

A DFT-based mechanistic study was performed in order to understand how the alcohols are able to reduce NO• to HNO, despite the unfavorable thermodynamics inferred from the corresponding reduction potentials.[13] Having ruled out a possible role for oxygen and/or metal ions and given the observed first-order kinetics on each reactant, we set out to analyze the direct reaction of NO with aromatic alcohols, namely, Tyr and $AscH^-$. The proposed mechanism (Scheme 4) starts with a PCNA of the −OH (or $-O^-$) group to NO•, which yields a radical intermediate, RO−N(H)O•, which, after O−N bond cleavage, releases HNO and an alkoxyl radical RO•.[13]

Scheme 4. Proposed Mechanism for the Reaction of NO• with Aromatic Alcohols[a]



[a]Upper panel: initial step of PCNA shown by the simultaneous nucleophilic attack (NA) and proton transfer (PT). The first HNO-releasing step, operative for all alcohols, produces the alkoxyl radical RO•. In the case of monoalcohols, the RO• radical reacts with a second molecule of NO• to produce a nitroso compound.

Depending on the alcohol, the alkoxyl radical can react either with another radical, like in the case of tyrosine to yield dityrosine, or with a second NO• to yield an *O*-nitroso compound, like for diols such as ascorbate or hydroquinone. The *O*-nitroso compounds were experimentally observed by cryogenic mass spectrometry.[13] *O*-Nitroso compounds derived from diols such as ascorbate and hydroquinone (which are two-electron reductants) are unstable, in contrast with the stability

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observed for aliphatic *O*-nitroso compounds like *tert*-butyl nitrite,[11] and can release a second molecule of HNO, resulting in two-electron-oxidized organic products. The energy barrier for breaking the O−NO bond corresponding to *tert*-butyl nitrite is 70 kcal/mol, whereas for *O*-nitrosoascorbate, it is only 14 kcal/mol, showing the difference in stability between both compounds.

The production of N_2O by HNO dimerization (or by the reaction of HNO with NO•), having a strongly negative ΔG°, is the driving force that assures that the thermodynamically unfavorable initial steps are pushed forward toward product formation.[13]

The DFT calculations for the corresponding key steps of the model compounds, presented in Table 2,[13] show that the first

Table 2. Calculated Reaction Energies (ΔE, kcal/mol)[13]

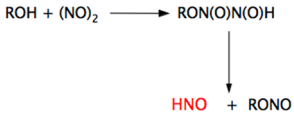
	ΔE_{PCNA} [a]	$\Delta E_{HNO\ first\ release}$ [a]	$\Delta E_{steps\ 1+2}$ [a]	global [a,b]
$AscH^-$	+16	−5	+11	−112[c]
HQ	+18.5	−10.5	+8	−109
Y	+25.4	+ 7.4	+33	−63
PhOH	+25.3	+12.4	+37.7	−70

[a] ΔE°_{PCM} (kcal/mol), electronic energies obtained from optimization at the B3LYP level using 6-31G(d,p) for all atoms using water (PCM = polarizable continuum model). Step 1: PCNA. Step 2: HNO first release. [b] The final product was DHA, BQ, *p*-Ph(OH)NO, and *o*-YNO, respectively; [c] In this case, we considered the global equation: AA + 6NO → DHA + $2N_2O$ + 2HNO.

PCNA step is endergonic, even considering the first HNO release. However, the thermodynamically favorable subsequent radical reactions, as well as HNO dimerization, and the reaction of HNO with NO• drive the global reaction forward. To gain further insight on reaction kinetics, we also computed the energy barrier for rupture of the O−N bond corresponding to the radical intermediate, which leads to the first HNO release. For ascorbic acid, the barrier is only 0.82 kcal/mol, whereas for phenol, the barrier is of 9.85 kcal/mol (see the Supporting Information for the energy profiles), following the observed trend in the apparent rate. Moreover, global analysis suggests that the endergonicity of the first step correlates with the bimolecular kinetic rate (Table 1, k_{app}), suggesting that the energy required to reach the RO−N(H)O• radical intermediate is a good estimate of the first, and key, step barrier.

An alternative to PCNA of the alcohol to NO would be the electrophilic attack of an NO dimer to the alcohol.[45,46] We analyzed this possibility for Tyr, and indeed an intermediate O−N(O)N(O)H (Scheme 5) can be formed, with a required

Scheme 5. Proposed Intermediate RON(O)N(O)H



energy of 17.7 kcal/mol, which can decay to yield HNO. This value is smaller than that for PCNA (25.4 kcal mol), but it has to be taken into account that the concentration of $(NO)_2$ is expected to be very low.

Another alternative mechanism could involve autocatalysis, especially in the case of the diols. The mechanism for HQ is shown as an example in Scheme 6. If a small amount of free radical is produced from the diol by the adventitious presence of minusculous amounts of dioxygen (O_2) or metals, HNO could be produced by H-atom abstraction of NO• from the alkoxyl radical [reaction (a)], followed by the reaction of HNO with NO• to produce the reactive HN_2O_2• (or its anion). The reaction of HN_2O_2• with HQ would regenerate the alkoxyl radical [reaction (b)]. However, reaction (a) competes with formation of the nitrite ester [reaction (c)] and reaction (b) with the fast reaction (d), already proposed by Lymar and co-workers.[47] Moreover, although this autocatalytic mechanism does not produce nitrite, in our previous experiments nitrite was found as a product in a practically equimolar ratio with respect to N_2O, suggesting that reaction (d) is the operative pathway.[13]

Going back to the key [−O−N(H)O]• radical intermediate, it is interesting to note that it is stable only if a proton is bound to the N atom and decays spontaneously back to the alcohol and NO• if the proton is not bound. We computed the energy for O−NO bond breaking in two different protonation states: ONOH or ON(H)O. In the first case, the products of this reaction were the alcohol and NO• with an energy barrier of 28 kcal/mol for the phenol, but it resulted in the formation of HNO and the phenoxyl radical with a 9.8 kcal/mol barrier for the second case. This fact remarks the relevance of proton transfer, either intramolecular or through the solvent, which together with the energetic data allows the following picture to emerge: electron-rich −OH or O^- groups are able to attack the relatively poor electrophile NO• thanks to the concomitant binding of H^+, which forms an electrophilic "$HNO^{\bullet+}$". This type of mechanism, PCNA (nucleophilic attack coupled to proton transfer), could be operative in many reactions involving weak nucleophiles and/or electrophiles.

The so-produced [O−N(H)O]• adduct now has a newly formed O−N bond, which is weak and will tend to break, keeping all electrons paired on the alcohol (yielding back the reagents NO• and ROH), or can break in a reductive manner, transferring an electron to the NO• group, resulting in HNO and the alkoxyl radical. The first equilibrium that goes back to the reagents is reversible, but the reaction that produces HNO is driven to products because of the high reactivity of HNO toward NO and itself, as well as that of the alkoxyl radical.

In summary, apart from this irreversibility, the other two factors that favor HNO production are the N-proton affinity and the stability of the alkoxyl radical. The first fact ties the electron to HNO, while the second one promotes electron transfer. Therefore, as evidenced by the kinetic/energetic trends, those alcohols that are known to form stable radicals are able to reduce NO• easier. On the contrary, aliphatic alcohols (such as sugars), which yield unstable radicals, will not reduce NO• because they do not produce the alkoxyl radical or otherwise rapidly decay back to the alcohol and NO•.

Finally, given the above-mentioned similar reactivity observed with thiols (see below), we also computed the first two reaction steps (PCNA and S−HNO• bond breaking) for thiophenol. The results show that in this case the energy required to yield the PCNA radical intermediate Ph−SHNO• product is 7.6 kcal/mol, thus considerably smaller than that observed for Tyr. Interestingly, the barrier for breaking the S−N bond to yield HNO is 29.3 kcal/mol, which is a large value compared to O−N bond breaking. This fact goes in favor of the originally proposed mechanisms previously shown in Schemes 1 and 2. However, in the case of thiols, after the first PCNA step, other mechanisms such as those involving disulfides and polysulfides could be operative.

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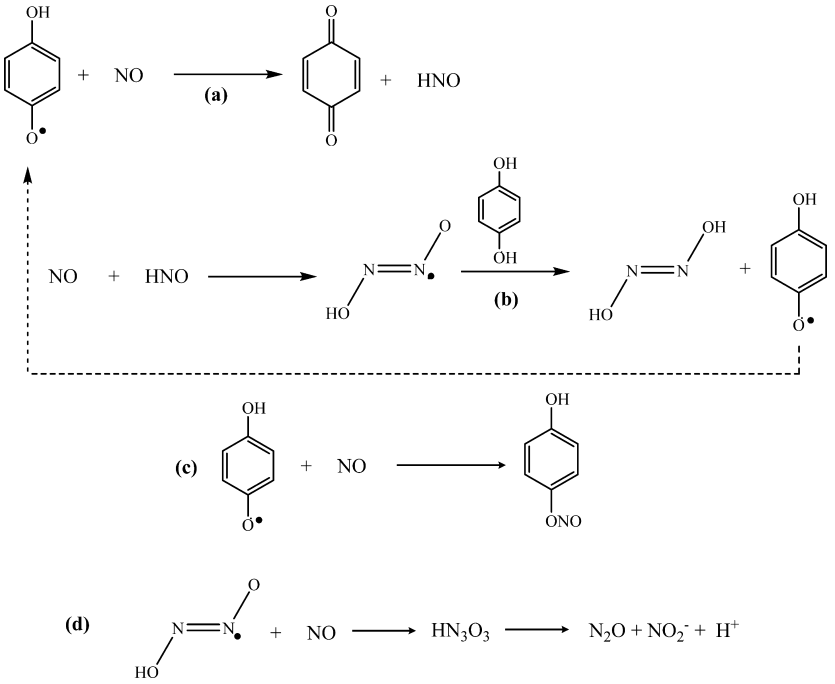
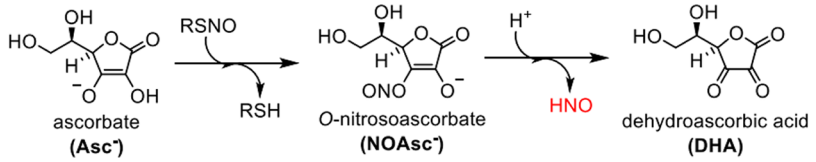
Scheme 6. Proposed Autocatalytic Mechanism for HNO Production



Scheme 7. Production of HNO from RSNO and Ascorbate



6. EFFECT OF O_2

The effect of oxygen in the reactions involving NO and thiols or other species has been a matter of discussion for many years. Under its presence, very reactive oxides such as NO_2 and N_2O_3 are produced, and in many cases reactions attributed to NO were indeed promoted by these oxides. However, HNO is a reduced product derived from NO, so it is difficult to understand why its production would be aided, accelerated, or catalyzed by O_2.

Anyhow, it is important to note that all of the reactions conducted in this and our previous work[13] were run under strict anaerobic conditions. In our previous work,[13] tests were conducted by adding controlled amounts of O_2. Under those conditions, it was observed that HNO formation is inhibited, but not completely suppressed, by O_2. The presence of O_2 diminishes the observed signal, a fact that can be attributed to its known reaction with $NO^\bullet$ to produce NO_2 and N_2O_3 and also to the reaction of O_2 with HNO, as shown before.[48] In conclusion, this is not an unexpected result, considering that HNO is a *reduced* product of $NO^\bullet$, while O_2 is an *oxidizing* agent.

7. BIOLOGICAL EXPERIMENTS

Given the moderately slow apparent rate and possible competing reactions, it is important to analyze whether alcohol/thiol-mediated NO-to-HNO conversion occurs in a complex biological system. In this respect, evidence so far comes from three different cases. Recently, the formation of extracellular HNO by the addition of $AscH^-$ or HQ was observed with NO-producing cells.[13] On the other hand, when a selective HNO fluorescence sensor (CuBOT1) was used to evaluate the intracellular HNO formation,[49−52] a clear increase of the fluorescence was observed.[13] These results indicate that HNO is indeed produced inside the cells by the reaction of endogenous $NO^\bullet$ with added $AscH^-$. In the same way, it was recently shown that HNO could be formed in a biological process, studying the reduction of intracellular *S*-nitrosothiols (RSNO) by ascorbate (Scheme 7) in incubated cells.[53,54]

Finally, in 2014 Filipovic and co-workers[55] have shown that the gasotransmitter H_2S may transform endogenous NO to HNO, which activates the HNO−TRPA1−CGRP cascade, suggesting broad physiological relevance of the findings. It is plausible that H_2S-mediated generation of HNO in vivo could be additionally related to its reaction with *S*-nitrosothiols,[56] as well as with metal nitrosyls.[57]

Although the results obtained from a cell culture show that NO-producing cells are able to yield HNO when loaded with ascorbic acid, whether it reacts directly with free NO or other NO-derived reaction products, such as nitrosothiols, which could also lead to HNO, is at this point impossible to differentiate. In any case, ascorbic acid, as well as other natural reductants, is very likely involved in the NO/HNO biological interconversion in a direct or indirect manner.

8. WIDER IMPLICATIONS OF NO/HNO BIOLOGICAL CONVERSION

After almost 3 decades of research, a wide spectrum of $NO^\bullet$ physiological roles have been uncovered, and their underlying

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mechanism is intensively interrogated. Among the most important are vascular tone control, immune response, and neurotransmission, as represented by the three $NO^{\bullet}$ production enzyme (NOS) isoforms. NO also has been shown to play important roles in cardiovascular and inflammatory diseases, cancer, and injury repair, opening several possibilities for its use as a therapeutic agent. Instead, HNO, or more precisely its donor compounds, have been mostly viewed as potential pharmacological compounds because they have overlapping but distinctive, sometimes preferable, effects compared to $NO^{\bullet}$ donors, but the physiological role of HNO is still being studied. Endogenous HNO, produced in vivo with a specific purpose, is until now just a hypothesis. The present data, together with our recent results,[13] may contribute changes in this paradigm, suggesting that $NO^{\bullet}$ can be converted to HNO by common biological mild reductants.

The two main questions that the present results put forward are thus the following: What are the most likely physio/pathological conditions for $NO^{\bullet}$-to-HNO conversion to take place? Which effects usually attributed to $NO^{\bullet}$ could be indeed mediated by HNO?

From a general redox perspective, it is clear that oxygen-rich and oxidizing environments will promote $NO^{\bullet}$ conversion to nitrite and nitrate, as catalyzed by oxyhemes, as well as other oxidative RNOS such as peroxynitrite, NO_2, and N_2O_3. On the other hand, hypoxic and reducing environments are expected to lead to HNO. More specifically, $NO^{\bullet}$, for example, has been shown to have significant effects in mitochondrial respiration, mainly through the partial inhibition of cytochrome *c* oxidase (CCO).[58] Inhibition of CCO, interestingly, could lead to accumulation of the reduced quinone pool, creating an ideal situation for $NO^{\bullet}$-to-HNO conversion, especially if oxygen tension is low. Also noteworthy are the proposed roles of myoglobin and other heme proteins, which in the absence of oxygen can reduce the abundant nitrate ion to $NO^{\bullet}$, producing it in an hypoxic environment, where its conversion to HNO could proceed using the available pool of circulating antioxidants, such as vitamin C. In other words, arginine and oxygen-independent $NO^{\bullet}$ production in or close to mitochondria are prime suspects for HNO production.

Also, $NO^{\bullet}$-to-HNO conversion in living organisms could be affected by their exposure to light. The interplay between the redox state of a living organism and its circadian rhythm has long been known. As an example, an endogenous antioxidant related to the circadian rhythm is the hormone melatonin, secreted by the pineal gland. Its levels increase dramatically during dark hours, and it scavenges free radicals and also stimulates antioxidant enzymes.[59,60] Therefore, when a human wakes up in the morning, its redox state is expected to be "more reduced" than that before sleep. This could translate into a more favorable environment for $NO^{\bullet}$ reduction in the morning and, consequently, a higher concentration of endogenous HNO.

Another ideal condition for in vivo HNO production could be induced pharmacologically by the joint incorporation of $NO^{\bullet}$ promoting or releasing compounds (like L-Arg) with common aromatic alcohol bearing drugs (i.e., aspirin) or foods rich in antioxidants (like vitamin C). For example, it has been observed that, in patients with coronary arterial disease, vitamin C augments arginine-induced vasodilation.[61] As mentioned before, arginine and aspirin have synergic effects on myocardial recovery.[14] Moreover, NO-releasing aspirin has shown distinct combined properties.[62] Concerning food, several works have shown that vitamin C and vitamin E as well as other antioxidants boost $NO^{\bullet}$ effects.[63] Ignarro and co-workers have shown that the antioxidants present in pomegranate juice enhance the biological actions of nitric oxide.[61,64] Many of these synergistic and distinct effects could be explained by in vivo $NO^{\bullet}$-to-HNO conversion. This suggestion brings us to the second question related to the biological effects of azanone: Which effects usually attributed to $NO^{\bullet}$ could be or are indeed mediated by HNO?

The results presented so far show that the so-called "unlikely" $NO^{\bullet}$-to-HNO conversion is possibly a commonly occurring reaction. The fact that several different aromatic alcohols are able to react point to a "common" underlying mechanism. However, as will be shown below, there is a moderate kinetic heterogeneity, which suggests that there is more to it than bearing an aromatic alcohol. The rates obtained can also be used to estimate a likely range of in vivo HNO concentrations derived from the reaction of $NO^{\bullet}$ with these compounds. Assuming that the physiological concentrations range of $NO^{\bullet}$ are from as low as 10 nM to as high as 150 μM in certain inflammatory diseases[65−68] and knowing that these pharmacological compounds display a range of 10−2500 μM in vivo circulating concentrations, this would result in HNO produced at picomolar to low nanomolar levels, which are large enough to produce a biological response. Under such conditions, at relatively low concentrations of $NO^{\bullet}$ and relatively high concentrations of antioxidants (such as ascorbate), competing reactions that consume HNO, such as dimerization or reaction with $NO^{\bullet}$, would not be operative, and, moreover, the concentration of O_2 would be at a minimum level because of its consumption by the antioxidants. Despite the fact that the analyzed situations may correspond to pathological and not to normal physiological cases, at this point a final conclusion cannot be established. We expect that, by opening the discussion, the chemical and biological community will be motivated to run the proper experiments that will allow a clear panorama to be attained.

Further complicating the picture, NO-to-HNO conversion has been also suggested to be mediated by thiols. We have recently shown in collaboration with other groups that HNO is able to activate specific transient receptor ion channels through the formation of disulfide bonds.[55] Disulfide bond formation is a usual outcome of thiol−HNO interactions, and it is significantly different from that of $NO^{\bullet}$, which in the presence of oxygen yields nitrosothiols. Which other biological processes rely on HNO−thiol interactions still needs to be addressed, and it is important to note that both $NO^{\bullet}$ and nitrosothiols could lead to HNO under certain conditions, making it a difficult task to separate $NO^{\bullet}$ from HNO-mediated effects.

Also, it is interesting to look for potential HNO targets in those of its older sibling. The $NO^{\bullet}$ main biological target is soluble guanilate cyclase (sGC), and its $NO^{\bullet}$-sensing capacity relies on its binding to a ferrous heme. HNO can also activate sGC, although, strikingly, in the ferrous and not the expected ferric state and to a lesser extent than NO.[69] The underlying mechanism is far from clear; it has been found that HNO activates an active site cysteine, and thus thiol-related chemistry has been suggested.[70] Thus, given the equilibrium between the ferrous and ferric states, HNO−sGC-mediated effects cannot be discarded.[71] Other $NO^{\bullet}$ targets are metalloproteins, such as CCO, but here again HNO displays significant overlapping reactivity, thus preventing a clear distinction between the NO and HNO mechanisms of action. Even neuronal NO synthase

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has been shown to produce HNO in the absence of a tetrahydrobiopterin cofactor.[72]

9. CONCLUDING REMARKS

In summary, HNO could be a new endogenously produced messenger that mediates specific physiological responses, many of which were attributed to direct $NO^{\bullet}$ effects. The underlying mechanism of this $NO^{\bullet}$−HNO interplay and its downstream effects in different contexts are still an open issue, where the basic chemistry of these compounds should play a major role. There also remain considerable gaps in our knowledge regarding the role of NO in vivo, particularly in humans. Therefore, what we do know is overshadowed by what we do not know. It becomes necessary to further investigate the mechanisms involved in $NO^{\bullet}$-to-HNO conversion by alcohols, thiols, and other molecules in the absence and presence of possible biologically relevant targets and also to perform measurements of HNO in cells, tissues, or organs in different chemical/biological conditions, for which improved sensing methods should be developed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.5b01347.

Experimental details and results including plots of HNO-selective electrode signal versus time and estimated energy profiles (PDF)

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Notes

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