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Nitric Oxide Is Reduced to HNO by Proton-Coupled Nucleophilic Attack by Ascorbate, Tyrosine, and Other Alcohols. A New Route to HNO in Biological Media?

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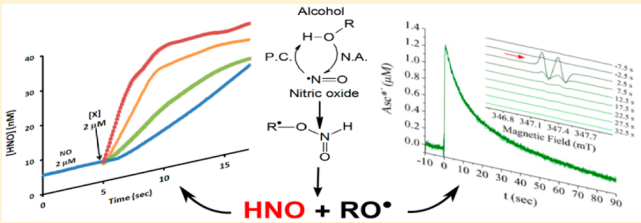
Supporting Information

ABSTRACT: The role of NO in biology is well established. However, an increasing body of evidence suggests that azanone (HNO), could also be involved in biological processes, some of which are attributed to NO. In this context, one of the most important and yet unanswered questions is whether and how HNO is produced in vivo. A possible route concerns the chemical or enzymatic reduction of NO. In the present work, we have taken advantage of a selective HNO sensing method, to show that NO is reduced to HNO by biologically relevant alcohols with moderate reducing capacity, such as ascorbate or tyrosine. The proposed mechanism involves a nucleophilic attack to NO by the alcohol, coupled to a proton transfer (PCNA: proton-coupled nucleophilic attack) and a subsequent decomposition of the so-produced radical to yield HNO and an alkoxyl radical.



INTRODUCTION

After over two decades of intense research, the chemical reactivity of nitric oxide and its key roles in several biological processes, including cardiovascular regulation, immune response, and neuronal physiology are, in principle, well established.[1−4] Azanone (HNO/NO$^-$), also called nitroxyl, is the one electron reduction product of NO and its reactivity and biological relevance are currently under intense debate.[5−8] It dimerizes rapidly ($k_{dim} = 8 \times 10^6$ M^{-1} s^{-1}),[9] which limits its concentration and lifetime in the solution. Moreover, HNO reacts quickly with its sibling NO ($k = 5.6 \times 10^6$ M^{-1} s^{-1})[10] and at a moderate rate ($k = 3 \times 10^3$ M^{-1} s^{-1}) with oxygen.[9,11,12]

HNO signaling is distinct to that of NO: HNO reacts mainly with thiols[8,13] and heme Fe(III) centers.[14,15] Unlike NO, HNO activates HNO-TRPA1-CGRP signaling cascade for the regulation of blood pressure and control of cardiac contractility.[5] The lack of certainty concerning its endogenous production is directly related to its elusive nature and the difficulties surrounding unequivocal and quantitative detection, especially when NO is present.

In the past decade several methods[16−23] have been developed allowing detection and quantification of azanone with discrimination from NO and other reactive nitrogen and oxygen species, RNOS. These methods include chemical trapping and HPLC product characterization,[17] UV−vis,[19−21] and fluorescence[22,24−26] detection and electrochemical detection.[27−29] In particular, our group has developed both a UV−vis trapping-based detection method that uses manganese porphyrins (MnP) and an azanone sensing electrode that is able to provide time-resolved quantification of HNO at the low nanomolar level.[21,28]

In a broader sense, the biological relevance of nitroxyl has at least two important aspects. The first concerns the studies of the pharmacological effects of HNO and the elucidation of the similarities with and the differences from NO.[30−34] The second is related to the possibility of its endogenous production as a biologically relevant messenger,[31] an intermediate metabolite, or an undesired enzymatic side product.[35−38] In this context,

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several in vivo azanone sources have been proposed. For example, HNO production could result from the activity of nitric oxide synthase (NOS) in the absence of the redox cofactor tetrahydrobiopterin.[36,38−41] Another well established in vitro enzymatic azanone source relies on the oxidation of hydroxylamine and other amino alcohols. Several groups have shown that this reaction can be catalyzed by heme-proteins like peroxidases, catalases, or even myoglobin.[17,42] On the other hand, chemical (nonenzymatic), biologically compatible routes to HNO have been, to our knowledge, much less pursued.[31,43] The most direct route, chemical reduction of NO, has been historically discarded, possibly due to the reduction potential of −0.8 V for the ($NO/^3NO^-$) couple, which is outside the biological range. However, at physiological pH, ^1HNO is expected to be the main species (pK_a = 11.4),[9] displaying an estimated $E°$ (NO, $H^+/^1HNO$) ≈ −0.14 V.[9,44] Moreover, it is important to note that the reduction of NO to HNO (reaction 1) could be driven forward by coupling with subsequent thermodynamically favorable reactions, such as N_2O production (reaction 2) or reactions between radical intermediates (reaction 3).

$$NO + ROH \rightarrow RO^{\bullet} + HNO \quad (1)$$

$$2NO + HNO \rightarrow N_2O + NO_2^- + H^+ \quad (2)$$

$$RO^{\bullet} + NO \rightarrow RONO \quad (3)$$

Interestingly, our recent results showed that HNO can be produced in vivo by the reaction of NO[5] or the nitrosyl species[30,45,46] with H_2S ($E°'(S^{\bullet-},2H^+/H_2S) = E°'(S^{\bullet-},H^+/HS^-)$ = 0.92 V at pH 7).[45] Also noteworthy, are several older works which showed that NO rebinds with generated $H^{\bullet}$ to yield azanone.[47−50]

In this work we demonstrate that NO can actually be reduced to azanone by several biologically relevant compounds bearing the −OH functional group resulting in a novel potential pathway for endogenous production of HNO.

■ EXPERIMENTAL SECTION

Reagents. Mn(III) 5,10,15,20-tetrakis(4-carboxyphenyl)-porphyrinate was purchased from Frontier Scientific and used as received. All reagents were purchased from Sigma-Aldrich and used as received. Trioxodinitrate ($N_2O_3^{2-}$) was synthesized according to published literature procedures.[29,51,52] Milli-Q grade water was used in all experiments; nitrogen and argon of high purity were used for anaerobic experiments. NO was generated anaerobically by dropwise addition of degassed water to a mixture of 4 g of $NaNO_2$, 8.5 g of $FeSO_4$, and 8.5 g of NaBr. The so-produced NO was passed through a NaOH solution to remove higher oxides and bubbled into degassed water in order to get a saturated solution of NO ([NO] = 2 mM).

Optical Absorbance. Measurements were recorded using an HP8453 spectrophotometer in 1 cm path-length quartz cuvette and using as blank the respective buffer solutions. All experiments were performed at 25 °C in 0.1 M phosphate buffer, pH 7.4, containing DPTA 10^{-4} M to avoid interferences or undesired reactions by Cu^{II} or other divalent cations. We also checked that all reactions were unaffected by the irradiation of the sample with the light source of the spectrometer.

Infrared Spectrometry. Spectra from 400 to 4000 cm^{-1} with 1 cm^{-1} resolution were recorded with a research series Thermo Nicolet FTIR spectrophotometer. All gas phase IR spectra were recorded using an 8 cm path length gas cell with NaCl windows. The IR spectrum of the N_2O present was quantified using calibration curves for the absorption bands showing peaks at 2212 and 2236 cm^{-1} for the P and R branches, respectively.[53] Under these conditions nitrous oxide signals for each injection were compared to a calibration curve prepared by injecting samples of N_2O produced in situ by NO_2−BSHA decomposition.[29] The detection limit for N_2O in the present conditions was 0.5 μmoles.

Amperometry. Measurements of HNO concentration were carried out with our previously described method based on a three-electrode system consisting of platinum counter electrode, Ag/AgCl reference electrode, and a gold working electrode modified with a monolayer of cobalt porphyrin with 1-decanethiol covalently attached. The method has been demonstrated to be specific for HNO, showing no interference or spurious signal due to the presence of NO, O_2, NO_2^-, and other RNOS.[27,28,53] Signal recording was performed with a TEQ 03 potentiostat.

In a typical experiment, 1.2 to 24 pmoles of ROH (0.2 to 4 μM) were added to 1.2 μmoles of NO dissolved in 6 mL (0.2 mM) of degassed distilled water containing 0.6 μmoles of DPTA (or EDTA) at room temperature (r.t.) under Ar atmosphere (or vice versa). For each case, we also confirmed that the maximum used concentrations (0.2 mM) of NO, and all $H^{\bullet}$ donors produced a very small signal that can be disregarded. We have also performed the reaction of NO with $AscH^-$ in an oxygen-free glovebox. In this case, water was deoxygenated by distillation under nitrogen atmosphere after addition of sodium dithionite. The results were very similar to those obtained with degassed water (Supporting Information, Figure SI3B).

Ion Chromatography. Measurements were recorded using a DIODEX DX-100 system, with an AS4A-SC (4 mm × 250 mm) column and an AG4A-SC guard column. The carrier was CO_3^{2-}/HCO_3^- 1.8/1.7 mM, with a flow rate of 2 mL/min

EPR Measurements. Solutions and buffers were prepared using high purity reagents and milli-Q grade water. All glassware was previously washed with HNO_3 and abundant milli-Q water and silicone tubing and plastic syringes were used to transfer solutions. Diethylene triamine pentaacetic acid (DTPA) (0.5 mM) and/or ethylene diamine tetraacetic acid (EDTA) (8 mM) were used as chelating agents to remove possible traces of catalytic metal ions. O_2 was eliminated from all solutions through vacuum-Ar cycles and a positive Ar pressure was maintained by bubbling Ar gas on the solutions throughout all handling.

For ascorbate anion, time scan experiments at a fixed magnetic field were also performed. The field $\mathbf{B}_0$ was chosen as the maximum of the low-field peak corresponding to the ascorbyl radical anion doublet. These experiments were performed with 1 G modulation amplitude, 6.33 mW microwave power, and a conversion time of 20 ms.

Computational Methods. To determine the reaction mechanism we performed DFT calculations using the Gaussian 98 software package. All involved species were optimized at the B3LYP level using 6-31 G(d,p) for all atoms using water (polarizable continuum model-PCM) in order to take into account solvation effects.

Mass Spectrometry. MS experiments were performed on maXis (Bruker Daltonics) ultrahigh resolution electron spray ionization time-of-flight mass spectrometer equipped with cryospray ionization module (Bruker Daltonics). Into 100 μM ascorbate solution in 80% acetonitrile/20% 10 mM ammonium carbonate buffer pH 7.4, 500 μM NO was added, and the reaction mixture was sprayed at −20 °C. Spectra were recorded over 15 min time.

Cell Experiments. Bovine Aorta Endothelial cell (BAEC, CLS Cell Lines Service GmbH, Germany) were grown in Ham's F12 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were loaded with CuBOT1 and fluorescence was recorded as previously described.[25,45] RAW 264.7 (mouse monocyte macrophage) from ECACC (Salisbury, UK) were grown in DMEM (Sigma-Aldrich, USA, cat. no. D5546) cell medium supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), 10% FBS (Sigma-Aldrich, USA), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 1% nonessential amino acid solution (Sigma-Aldrich, USA) in T-75 cell culture flask at 5% CO_2 and at 37 °C. Cells were stimulated with 1 μg/mL LPS (Sigma-Aldrich, USA) overnight and next day mechanically detached, washed once with HBSS with Ca^{2+} and Mg^2, and used immediately for analysis. We used 1 × 10^6 cells per sample in HBSS with Ca^{2+} and Mg^{2+} supplemented with different concentration of FBS (fetal bovine serum) up to 5%. The

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temperature of the HBSS w/o Ca^{2+} and Mg^{2} used in measurement experiments was 37 °C.

■ RESULTS AND DISCUSSION

Aromatic Alcohols and Ascorbate React with NO to Produce HNO. Our first approach to determine the possible production of HNO from the reaction of NO with aliphatic or aromatic alcohols was performed by measuring the conversion of Mn(III)TCPP to $\{MnNO\}^6$ (Enemark-Feltham notation) using UV–vis spectroscopy (see Supporting Information for more details).[54] Figure SI1A shows the absorbance changes obtained after mixing NO solution with ascorbate (AscH−), the predominant species under the reaction conditions. These changes are characteristic for the reaction between Mn(III) porphyrins and HNO, with the consequent formation of $\{MnNO\}^6$.[21] Since this Mn(III) porphyrin reacts neither with NO[21] nor with ascorbate[55] (see control experiments in Supporting Information, Figure SI1 and SI2) these results strongly suggest HNO production. Similar results were obtained with hydroquinone (HQ), tyrosine (Y), and phenol (PhOH), although the reaction rates varied significantly (see Table 1). No reaction was observed with nonaromatic alcohols like methanol, D-mannitol, or malic acid. The second approach used to determine HNO production relied on the recently developed HNO selective electrode, which allows time-resolved nanomolar detection.[27−29,53] In Figure 1 we present the amperometric signal versus initial time plot after the addition of each alcohol (2 μM) to an anaerobic aqueous solution of NO (0.2 mM). The increase in the current following the addition of the alcohol clearly proves the HNO formation. As expected for a bimolecular reaction, the signal peak, which reflects the HNO concentration,[28] is linearly dependent on both $AscH^-$ and NO concentrations (Supporting Information, Figure SI3).

Table 1. Amounts of N_2O and Nitrite Obtained for the Reactions of H• Donors with NO, and the Corresponding k_{eff}

compound[a]	k_{eff} (M^{-1} s^{-1})[b]	NO_2^- (μmol)[c]	N_2O (μmol)[c]	N_2O yield[c]	org. prod. yield[d]
$AscH^-$	8.0 ± 0.5 (43 ± 15)	20	16	50%	>95%
HQ	6.1 ± 0.4 (9)	11	9	30%	>95%
PhOH	3.2 ± 0.4	8	6	20%	∼ 90%
Y	0.9 ± 0.4	5	4	10%	∼ 30%

[a]No reaction was detected when methanol, D-mannitol or malic acid were used. [b]Determined from the slope of the electrode signal. Between parentheses, determined by EPR, see Supporting Information for details. [c]After 24 h, based on the initial amount of NO (100 μmol). [d]Dehydroascorbate (DHA), benzoquinone (BQ), *p*-Ph(OH)-NO, and *o*-Y-NO respectively, based on 17 μmol (initial amount).

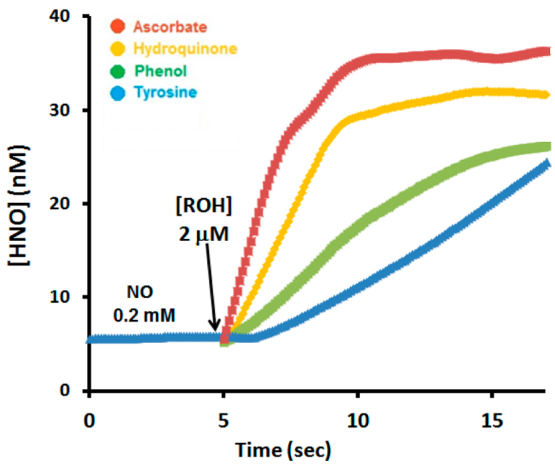

Figure 1. Amperometric signal vs initial time plot after the addition of 2 μM ROH to an anaerobic aqueous solution of NO (0.2 mM): *y*-axis, [HNO] after calibration. ROH = (red) $AscH^-$; (orange) HQ; (green) PhOH; (blue) Y.

Figure 2A and Supporting Information, Figure SI3C show that v_i (initial rate) versus [ROH] and [NO] plots are linear. From the slope of these plots an effective bimolecular reaction rate constant (k_{eff}), corresponding to reaction 1 can be obtained.

$$v = k_{eff}[ROH][NO]$$

The resulting k_{eff} are reported in Table 1, and the data show that both diols (HQ and $AscH^-$) react ca. 5−10 times faster than phenols, with $AscH^-$ being the fastest.

On the other hand, Figure 2B and Supporting Information, Figure SI3D show that the log(v_i) vs log[ROH] and log[NO] plots are linear with a slope close to 1, confirming that the reaction is first order in both reactants.

We also tested whether Fe(II/III), Mn(II), Cu(I/II), or Co(II) affected HNO production in the described reactions by using the electrochemical nitroxyl sensor. The results confirmed that metal ions do not play any significant role in the production of HNO (see Suppporting Information, Table SI2).

EPR Analysis. Since a formal H atom abstraction from −OH groups by NO would produce a free radical species, the reactions were studied by EPR. Ascorbate (0.2−2 mM), hydroquinone (10 mM), and tyrosine (2 mM) solutions were mixed with equal volumes of the NO saturated solutions by simultaneous rapid injection into a quartz flat cell. The presence of dioxygen and metal ions (DPTA or EDTA were used as chelators) was excluded. The first two alcohols produced clearly detectable EPR signals as shown in Figure 3 and Supporting Information, Figure SI5. Tyrosyl radicals were not observed, presumably due to the slower reaction rate between NO and Y and/or the lower stability of the tyrosyl radical. Figure 3 shows the time dependence of the ascorbyl radical concentration obtained after mixing $AscH^-$ and NO.

After mixing the reactants, an intense ascorbyl radical signal appears which subsequently decays with a half-life of 4−8 s. This behavior is consistent with disproportionation of the ascorbyl radical into ascorbate and dehydroascorbate,[56] and also reaction of ascorbyl with NO to give O-nitrosoascorbate.[43,57] For the reaction with HQ (shown in Supporting Information, Figure SI5) similar results were obtained, but the radical signal corresponding to HQ• increases 6-fold and remains stable for several minutes, slightly decaying after 15 min. The EPR signals also allow determination of the k_{eff} for both reactions (shown in Table 1). k_{eff} values obtained by EPR are in the same order of magnitude as those obtained from the electrochemical data.

The ubisemiquinione EPR signal has been reported during the reaction between NO and truncated ubiquinols,[58] and the ascorbyl radical has been observed during the reaction between ascorbate and N-acetyl-N-nitrosotryptophan or NO donors under normoxic and oxygen free conditions.[43] The kinetic

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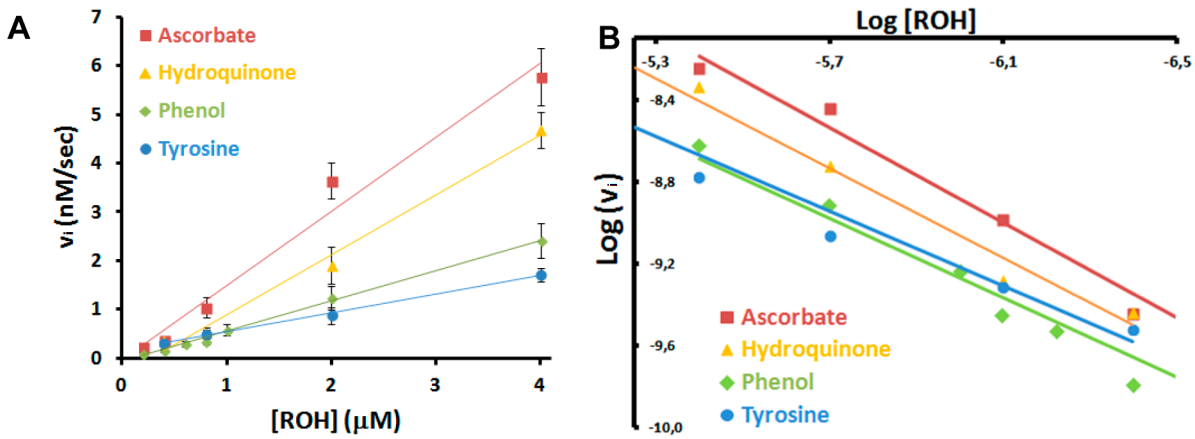

Figure 2. (A) v_i vs [ROH]. (B) log(v_i) vs log[ROH]. [NO] = 0.2 mM. ROH = (red) AscH$^-$; (orange) HQ; (green) PhOH; (blue) Y.

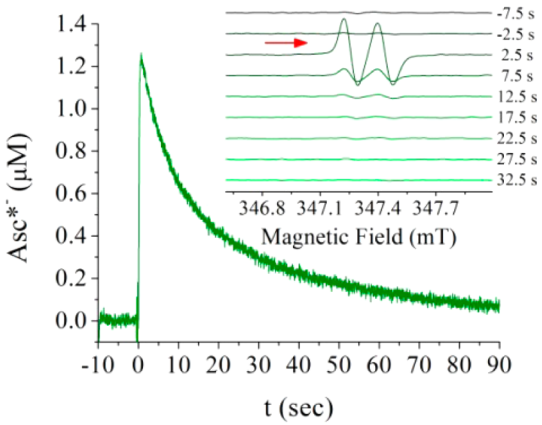

Figure 3. Time dependence of ascorbyl radical concentration. Inset: Consecutive EPR spectra of solutions of ascorbate (1 mM) alone and with NO (1 mM). The arrow indicates beginning of the reaction.

analysis of these reactions is detailed in the Supporting Information.

End Products Analysis. The initial products of the reaction of NO with the alcohols are unstable and highly reactive radical species. Thus, further reactions are expected to occur. The main sink for HNO is expected to be its dimerization and/or reaction with NO,[10] yielding the stable products N_2O and NO_2^-. To detect and quantify N_2O we determined the IR spectra of the reaction chamber headspace. As expected, NO reaction with HQ, AscH$^-$, Y, and PhOH results in the appearance of characteristic N_2O IR bands at 2210 and 2230 cm^{-1} (see Supporting Information, Figure SI7),[53,59] and no signal is observed with either reactant alone. The presence of nitrite was confirmed by ion chromatography (see Figure SI8). Moreover, quantification of the relative N_2O and NO_2^- yields (Table 1) show that they are formed in a ca. 1:1 ratio, which is consistent with our mechanistic interpretation (eq 9; vide infra).

The R-O$^\bullet$ radicals are also inherently unstable and thus react further leading to more stable organic closed shell compounds. To determine the corresponding end products for each reaction, we used NMR spectroscopy, IR, UV, and MS spectrometry (see Supporting Information). AscH$^-$ yields dehydroascorbate (DHA) as the main end product, formed by ascorbyl radical disproportionation. When studied by cryo-spray ionization ultrahigh-resolution mass spectrometry, the reaction of AscH$^-$ and NO showed MS peaks (m/z 207.0368, 223.0591 and 237.0378, Supporting Information, Figure SI9), which correspond to the first addition of NO to ascorbate, and second addition of NO to either first RO−NO$^-$, or the ascorbyl radical (see below for mechanistic analysis). As postulated by Kirsch,[43] once the nitrite ester [AscONO]$^-$ is formed by the reaction of Asc$^{\bullet-}$ with NO, HNO and DHA can be produced via a radical chain mechanism as shown in eq 4,[43] eq 5,[60] and eq 6.[43]

$$[\text{AscONO}]^- + \text{H}^+ \rightarrow \text{DHA} + \text{HNO} \quad (4)$$

$$\text{DHA} + \text{Asc}^- \rightleftharpoons 2\text{Asc}^{\bullet-} \quad (5)$$

$$\text{Asc}^{\bullet-} + \text{NO} \rightarrow [\text{AscONO}]^- \quad (6)$$

HQ yields mainly benzoquinone (BQ), also possibly due to further reaction of the HQ radical with NO. Finally, PhOH and Y yield the corresponding products 4-nitrosophenol (p-Ph(OH)-NO) and 3-nitrosotyrosine (o-Y-NO), whereas Y also dimerizes to yield dityrosine (see Supporting Information for experimental details); these products are consistent with the presence of PhO$^\bullet$ and Y$^\bullet$ radicals. The lack of EPR signal in these cases possibly arises because of their high reactivity and the presence of the excess of NO, which yields the mentioned products. The yields of the organic products (see Supporting Information for details) are higher than the corresponding N_2O yields, indicating that these compounds are also produced by other routes which do not afford HNO. The formation of nitrosocompounds by reaction of phenols with NO has been observed before.[61]

Computational Mechanistic Analysis. To get an additional insight into the reaction mechanisms we performed DFT calculations using the Gaussian software package. As an example, the results for AscH$^-$ are presented in Scheme 1, while the other cases are shown in Supporting Information, Figure SI11. The calculations show that the first step of the reaction between NO and AscH$^-$ is endergonic (by 16 kcal/mol) yielding a radical intermediate RO-N(H)O$^\bullet$ (consistent with one of the peaks observed in the mass spectrometer at m/z 207.0368, see Scheme 1 and Supporting Information, Figure SI9). This step can be described as a nucleophilic attack of the ascorbate anion to NO (reaction 7), coupled to proton transfer from the vicinal −OH moiety or the solvent. Such a mechanism can be described as a proton-coupled nucleophilic attack (PCNA).

NO binds preferably to C2−O, while ascorbate is preferably deprotonated at C3−O (see Scheme 1). At this point it is

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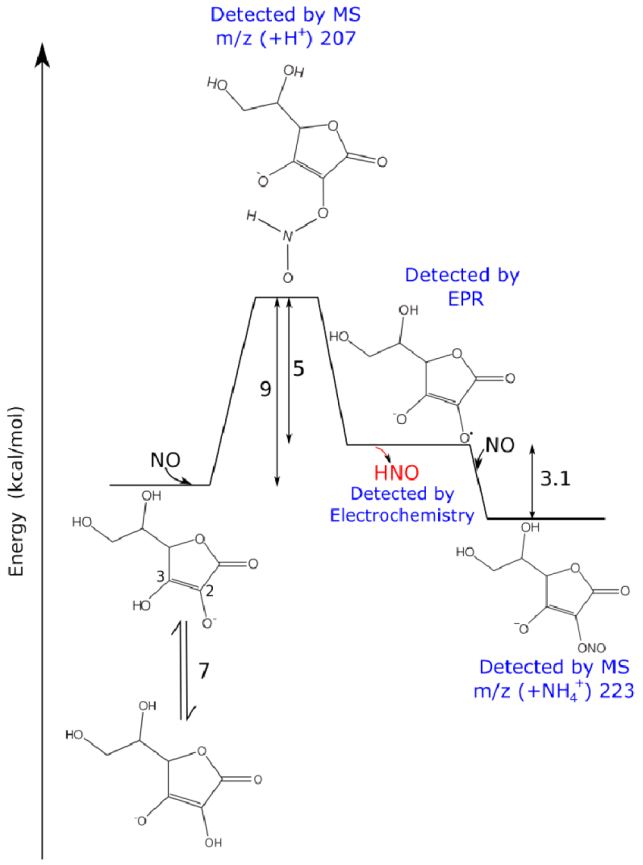
Scheme 1. DFT Calculations. Energy Values Reported in kcal/mol



difficult to determine whether the NO reacts with one or the other tautomer, with the OH or O^-, and how and when the protons are transferred. However, attack of -O^- to NO seems to be more likely. The RO-N(H)O$^\bullet$ radical intermediate decays to HNO and the ascorbyl radical (reaction 8), which can then react with another NO to produce a closed shell nitrite ester *o*-nitrosoascorbate (also observed by MS at *m*/*z* 223.0591). Reaction of the radical with the second NO prior to its HNO release, possibly accounts for formation of di-ONO observed by MS (*m*/*z* 237.0378, Supporting Information, Figure SI9g). The *O*-nitrosoascorbate also decays after taking a proton to yield HNO and DHA, as previously observed by Kirsch and co-workers.[57]

$$Nu^-(+H^+) + NO^\bullet \rightarrow (Nu - NH - O)^\bullet \quad (7)$$

$$(Nu - NH - O)^\bullet \rightarrow HNO + (Nu)^\bullet \quad (8)$$

A similar mechanism is expected for HQ (see Supporting Information), with two NO molecules reacting with each HQ molecule. For Y and PhOH, the radical intermediates produced after the addition of NO, formation of the RO-N(H)O intermediate, and HNO release also yield the observed nitroso derivatives. More importantly, taking into account the pK_a of the corresponding alcohol, in these three cases the reaction undoubtedly occurs with a neutral OH group, where an intramolecular proton rearrangement or solvent-assisted protonation is required. Therefore, in these cases a PCNA is proposed as well.

Last but not least, it is important to note that although the first reaction step between NO and alcohol is endergonic, the reaction is driven forward by the subsequent reactions of the initial products (HNO and radicals). In fact, HNO dimerization overcompensates the endergonic HNO generation resulting in an overall negative free energy balance for the global reaction 9 (see Scheme 1), which for $AscH^-$ is

$$AscH^- + 6NO \rightarrow DHA + 2N_2O + 2NO_2^- + H^+ \quad (9)$$

The energy associated with the first step, either to yield directly HNO by HAT or an "RON(H)O−like" radical intermediate by PCNA, can be considered a minimum estimation of the global reaction barrier. As shown in Table 2, the Δ*E* for the first two

Table 2. Ab Initio Calculated Reaction Energies (Δ*E*) in kcal/mol for PCNA and HNO Release Steps

	pK_a	$E^{\circ\prime}$ (V) (pH 7) RO$^\bullet$, H$^+$/ROH	Δ*E* PCNA[a]	Δ*E* HNO release[a]	Δ*E* step 1+2[a]	global[a,c]
$AscH^-$	4.11	0.28	+16	- 5	+11	−58
HQ	10	0.10	+ 18.5	10.5	+ 8	−109
Y	10	0.91	+ 25.4	+ 7.4	+33	−63
PhOH	10	0.97	+25.3	+12.4	+37.7	−70
MeOH[b]	15.5	-	+ 19.5	+ 33.7	+53.2	-

[a] ΔE°_{PCM} (kcal/mol), optimized at the B3LYP level using 6-31 G(d,p) for all atoms using water (PCM: polarizable continuum model); step 1, PCNA; step 2, HNO release. [b] HNO was not detected when methanol was used. [c] Final product was DHA, BQ, *p*-Ph(OH)NO, and *o*-YNO, respectively (see SI).

steps (step 1+2) are smaller for $AscH^-$ and HQ, which are the faster reactants (Table 1). The largest Δ*E* (+53.2 kcal/mol) is observed for MeOH, which does not react under the tested conditions. The calculated energies for step 1+2 and for the global reaction are in reasonable agreement with those energies obtained from tabulated redox potentials (Supporting Information, Table SI3).

To assess the potential role of molecular oxygen on these reactions, we performed the reaction of $AscH^-$ and NO in the presence of controlled amounts of oxygen (Supporting Information, Figures SI2 and SI4). As shown in Figure SI4, the amount of HNO produced decreases as the relative amount of added O_2 is increased. This is a strong indication that O_2 does not catalyze HNO formation. Instead, the presence of O_2 diminishes the observed signal, a fact that can be attributed to its known reaction with either reactant, or even with azanone, as shown in our previous work.[28]

In Vitro Cell Studies. In certain cell types, such as endothelial cells, neuronal cells, and immune cells, vitamin C accumulates to concentrations higher than 1 mM.[62] To analyze whether the described reactions occur under physiological conditions, we used an HNO fluorescence sensor, CuBOT1, to evaluate the intracellular azanone formation.[22,24−26] Bovine arterial endothelial cells were pretreated with either 1 mM $AscH^-$ or 1 mM pBQH2 for 1 h to increase their intracellular concentration. Cells were washed and then loaded with CuBOT1 to assess the changes in intracellular levels of HNO. The intensity of the fluorescence was compared with basal fluorescence detected in the control (untreated cells). Figure 4A shows a clear increase of the fluorescence with both treatments. In addition we tested the ability of ascorbate to reduce endogenously generated NO from another cell line, RAW 264.7 macrophages. Macrophages were stimulated with lipopolysaccharide(LPS)/interferon gamma to stimulate indu-

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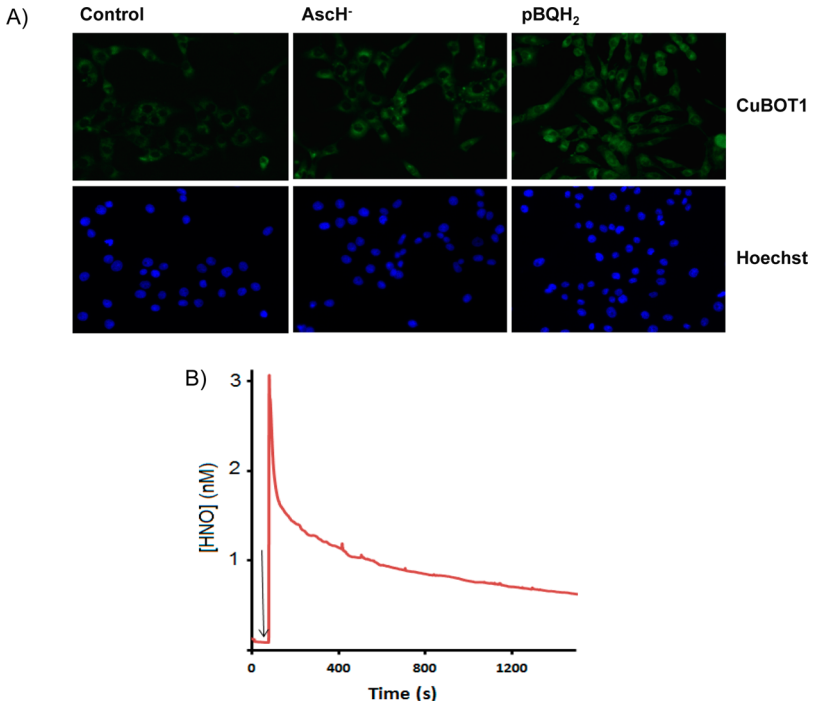


Figure 4. (A) Intracellular HNO formation in bovine arterial endothelial cells as revealed by the HNO fluorescence sensor, CuBOT1. Hoeschst was used to stain the nuclei, showing that there are cells in the control for which the signal is very low, and also that the position of the signal matches the actual cells. (B) HNO formation after the addition of ascorbate to immunostimulated macrophages. The HNO electrode was immersed into a 10^6 cell/mL suspension of immunostimulated macrophages in Dulbecco's Modified Eagle's Medium (DMEM). Subsequently 1 mM ascorbate was added, and the current was monitored.

cible nitric oxide synthase to produce NO, and the HNO electrode was immersed in the extracellular medium containing 10^6 cells/mL. After the addition of 1 mM ascorbate an immediate rise in the signal was observed, showing clear HNO formation (Figure 4B). No signal was observed when $AscH^-$ was added into cell-free medium.

These data strongly suggest that HNO could be produced in the reaction of NO and $AscH^-$ under physiological conditions.

■ CONCLUSIONS

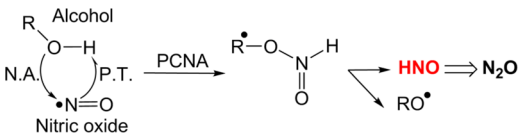
The present work provides clear evidence of a possible biochemically relevant HNO source, resulting from the reaction of NO with aromatic or "pseudoaromatic" alcohols such as tyrosine, ascorbic acid, and hydroquinone. Mechanistically, it is clear that the reaction does not involve a simple outer sphere reduction coupled to proton release/uptake, which is thermodynamically unfavorable as evidenced by the alcohol reduction potentials shown in Table 2.

Instead, our data suggest that there is a nucleophilic addition of ROH/RO^- to NO, coupled to a proton transfer (either intramolecular or through the solvent) that results in an RO-N(H)O$^\bullet$ intermediate, which decays by O−N bond cleavage, producing HNO and the corresponding radical (see Scheme 2 and Table 2). The stability of the RO$^\bullet$ radical (bound to HNO or free), PCNA endergonicity, and the global energy for steps 1 + 2 (Table 2) seems to be the key factor for the reaction to occur, explaining why no reaction is observed for MeOH or mannitol, and why $AscH^-$ and HQ react faster.

Beyond the chemical novelty, biological implications are direct. For example, given the known preference for NO partition within the hydrophobic interior of biological membranes[63] and its physiological role in plant and animal mitochondria, the following picture emerges:[64,65] under hypoxia, respiratory chain intermediate quinones accumulate and NO production increases, through nitrite reductase activity of myoglobin among others,[66] creating an ideal opportunity for the presented reaction to take place. In addition, the presented proof of concept for physiological NO conversion to HNO, suggests that it is not unlikely that some of the protective effects assigned to NO, are indeed mediated by its "younger" sibling HNO,[67] as shown in our recent work.[31]

Definitive proof to these hypotheses awaits further studies and opens the way for both potential therapeutic interventions of azanone donors and understanding of endogenous HNO production.

Scheme 2. Proposed Mechanism for HNO Formation by the Reaction of NO with ROH



■ ASSOCIATED CONTENT

Supporting Information

Kinetic analysis, ab initio calculations, mass spectra, EPR, and other experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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