Nitrite Anion: The Key Intermediate in Alkyl Nitrates Degradative Mechanism

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Alkyl nitrates are metabolized in vitro to yield nitric oxide, and thiol groups have long been considered necessary cofactors. Here, we report evidence that no reaction between thiols and alkyl nitrates takes place in vitro, but stronger reducing agents, such as iron(II) derivatives, are necessary; alkoxy radicals and nitrite anions are the reaction intermediates. The latter, in slightly acidic conditions, can nitrosate thiols to the corresponding *S*-nitrosothiols, the real NO releasers.

Introduction

The role of sulfhydryl derivatives has always been underlined by the effects of compounds such as *N*-acetylcysteine and glutathione on the hemodynamic responsiveness to nitrates, inducing a decrease in tone paralleling an increase in sulfhydryl derivatives concentration. In fact, the direct interaction of thiols and nitrates has usually been considered the source of NO, and only more recently an enzymatic pathway has been taken into account.[1–5] In particular, it has been hypothesized that P450, the enzyme most commonly involved in drug metabolism,[1] takes part in the biotransformation of organic nitrates, leading to the formation of NO[3,6] via a multi-electron mechanism. However, the latter hypothesis contrasts with other reports[7,8] that highlight the role of thiols mediated by the formation of the corresponding *S*-nitrosothiols.[9,10]

Results and Discussion

A definitive study on the role of thiols, i.e., if they act as direct reducing agents or if other reductants are responsible for the nitrate degradation, has never been reported. Thus, to investigate this topic, experiments between glutathione, cysteine or benzylthiol, and alkyl nitrates such as the 5-phenyl-4-pentene-1-nitrate and the 1-penten-5-nitrate were conducted. These experiments led to the discovery that the thiol and the nitrate were unchanged; i.e., no reaction took place. This result, definitely in contrast with those reported in the literature, seemed to indicate the inability of thiols to induce directly the nitrate reductive process. On the contrary, it suggested that a stronger reducing agent was necessary. In particular, the behavior of P450 in vivo[6] supported the hypothesis that iron(II) derivatives could succeed. Thus, experiments with $FeCl_2$ and the 5-phenyl-4-penten-1-nitrate or the 5-nitrate-1-penten, in anhydrous solvents, were conducted. As a matter of fact, the formation of **1**−**5** can be accounted for only through a radical mechanism (Scheme 1). In particular, the intermediate alkoxy radicals, via a 1,2- or a 1,5-exo ring closure process, led to the formation of carbon centered radicals, which are precursors of these species, are well known markers of radical mechanisms (Table 1).[11] These results proved the capability of iron(II) derivatives to induce the degradative nitrate reduction via an electron transfer but not which mechanism is involved (Scheme 2).

One of the most accredited hypotheses for the NO production, contrasting with that usually acknowledged,[7,8] highlights the role of thiols mediated by the corresponding *S*-nitrosothiols;[9] i.e., the degradative process conducted in the presence of thiols leads to the formation of the corresponding *S*-nitrosothiols. However, assuming a two-electron mechanism, NO should be formed immediately, and to perform the thiols' nitrosation, it should be oxidized to nitrous anhydride (N_2O_3).[12] But this oxygen-induced process has been shown to be irrelevant in vivo because it is negligibly slow.[13,14] In contrast, if a one-electron mechanism is considered, the intermediate nitrite anion itself could carry out the thiol nitrosation, a process that can take place also in very light acidic conditions (Scheme 2).[15,16]

To discriminate between these hypotheses, the 5-phenyl-4-penten-1-nitrate was first reacted with $FeCl_2$ in the presence of GSH in a neutral and aerated solution directly in the cell of a UV spectrometer. In this way the formation of the *S*-nitroso-glutathione could be directly detected through its UV absorption spectrum. But no GSNO was indicated. This result indicated against a two-electron mechanism, i.e., the direct formation of NO, and underlined the low probability of its oxidation to N_2O_3. In contrast, when the same experiment was repeated in anaerobic conditions and at pH 6.5, the formation of GSNO was clearly evidenced. In fact, the nitrite anion through its acidic equilibrium is in equilibrium with the nitrous acid and the nitrous anhydride, a well-known nitrosating species.[12] This result definitely confirmed the possibility of iron(II) derivatives to induce a nitrate reductive process via a monoelectron transfer, leading to alkoxy radicals and nitrite anions as intermediates.

Several troublesome aspects of organic nitrate ester therapy are known in vivo, and one of the most serious is that patients become refractory to its effect: tolerance. This decrease in NO production, concomitant to the decrease of thiols' level in tissue, depletion,[17] has been hypothesized to be affected by the enzyme activity.[3] In particular, tolerance seems to increase with the oxidation of the enzyme, as supported by the counteraction of supplied reducing agents.[10,18–23] Furthermore, the role of the enzyme is indirectly underlined by the absence of tolerance in NO releasing substrates that do not require enzymes.[24] To prove if an analogous behavior could be hypothesized also in vitro, experiments with nitrates and $FeCl_3$ in both the absence and in the presence of thiol derivatives were conducted. When experiments were carried out in the absence of thiols, no reaction took place. That stressed the impossibility for iron(III) derivatives (oxidized state) to interact directly with nitrates. In contrast, when the experiments were repeated in the presence of GSH, cysteine, or benzylthiol, the nitrate degradative reaction did occur.[25,26] In particular, from the reaction of 5-phenyl-4-penten-1-nitrate with $FeCl_3$ in the presence of GSH, products **1** and **6**

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Scheme 1. Electron Transfer Process: Radical Intermediates

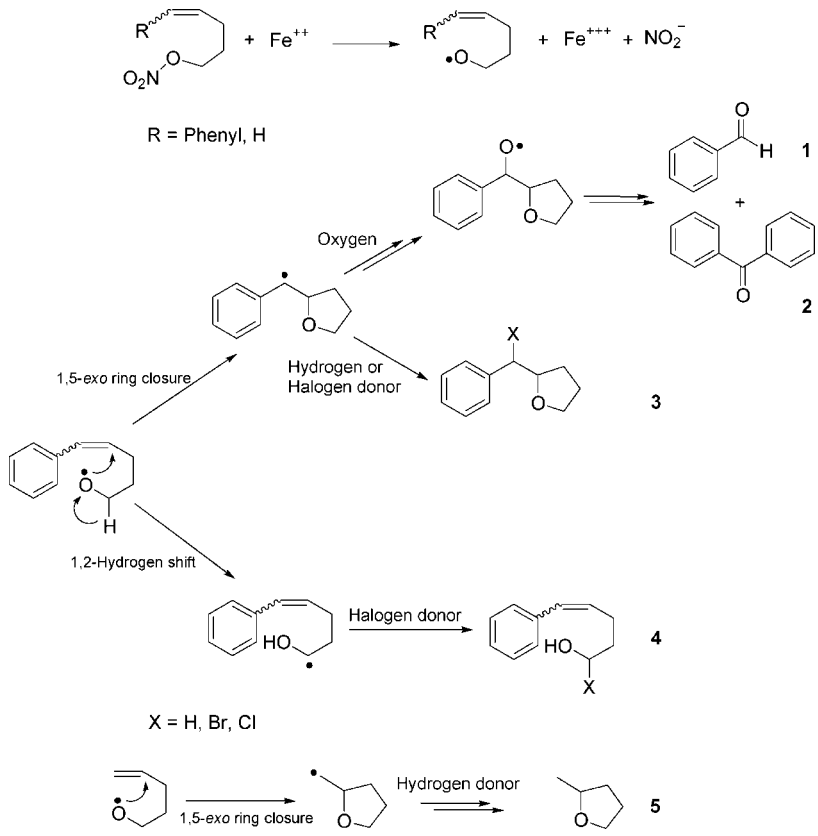
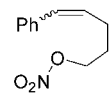
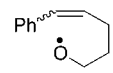
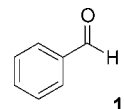
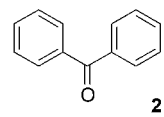
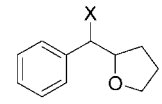
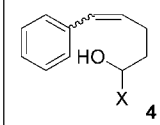
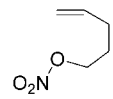
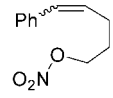
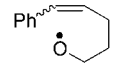
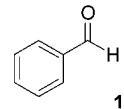
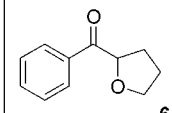


Table 1. Reaction Products: Markers of the Radical Process

	Organic Nitrate	Alkoxy Radical	Reaction Products			
Fe^{++}			 1	 2	 3 X = H, Br, Cl	 4 X= H, Br, Cl
					 5	
Fe^{+++} + GSH			 1		 6	

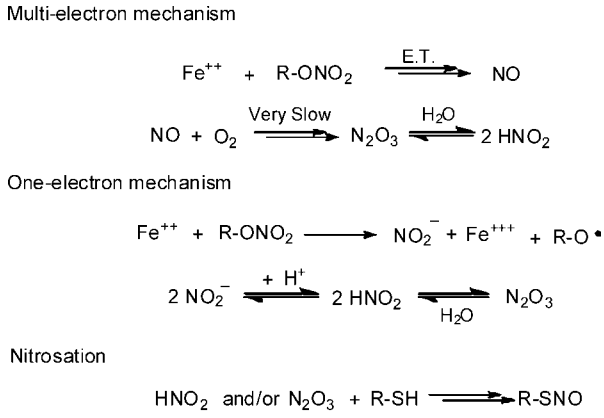
were detected, species corresponding to those obtained when the 5-phenyl-4-penten-1-nitrate was reacted with $FeCl_2$ alone (Table 1). This confirmed the role of iron(II) in the nitrate reductive process, and its formation is accounted for via the reduction of iron(III) carried out by reducing species such as thiols. The role of antioxidants (thiols) was also stressed by results obtained in experiments conducted at steady concentration of nitrate and variable thiol/iron(III) molar ratios; these showed the highest nitrate degradation yield when a 2.5:1 thiol/iron(III) molar ratio was used, i.e., conditions in which the conversion of iron(III) into iron(II) is favored (Scheme 3).[27,28]

These in vitro results led to the hypothesis of a parallel behavior of P450 in vivo. In fact, in the cell, the P450-iron(II), acting as nitrate reducing agent, turns into P450-iron(III), but in order for the enzyme to continue its function, the P450-iron(III) must be reconverted into P450-iron(II), and this can be performed by thiols. In fact, cysteine and GSH present in the cell in high concentration could succeed.

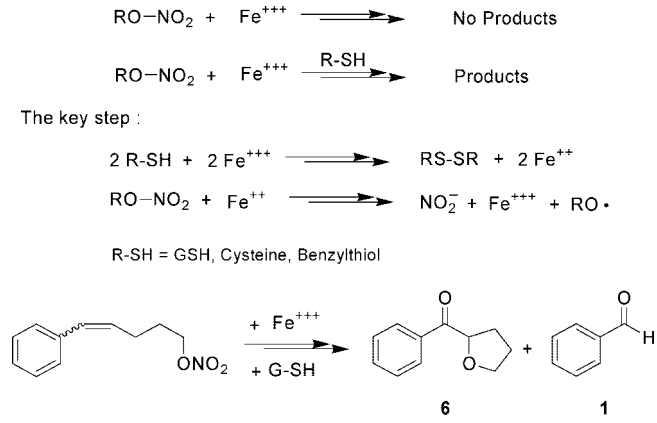
Conclusion

The degradative NO release from alkyl nitrates starts from a one-electron transfer process between the nitrate, the oxidant,

Scheme 2. Mechanism of Degradation of Nitrates



Scheme 3. Reduction of Nitrates: Role of Thiols



and an iron(II) derivative, the reductant. This mechanism is supported by experiments that led to the detection of products that unquestionably sustained a radical mechanism, i.e., that can be accounted for only via an electron transfer process. The nitrate degradative process, besides alkoxy radicals, led to the formation of nitrite anions, an inactive NO derivative, which in slightly acidic conditions, for example, like in ischemic conditions, is able to induce the nitrosation of thiols to the corresponding *S*-nitrothiols, the real NO suppliers. These results led to a hypothesis of a parallel behavior with P450, i.e., the role of the iron(II)/iron(III) redox couple and thiols inside the cell, in support of the intermediacy of *S*-nitrosthiols. It follows that intracellular thiols, in particular cysteine and GSH, being involved in the fundamental processes, will decrease in concentration over time. This accounts for depletion and for less efficiency in re-establishing the enzyme reducing capability, tolerance.

Experimental Section

The 5-phenyl-4-penten-1-nitrate was synthesized from the parent 5-phenyl-4-penten-1-ol as reported[29] and reacted with *N*-bromosuccinimide to lead to the 5-phenyl-4-penten-1-bromo, which under the action of silver nitrate leads to the nitrate. The 5-nitrate-1-pentene was obtained by reacting the 5-bromo-1-pentene, commercial grade, with silver nitrate. All the other reagents were commercial products and used as received. In particular, solvents such as acetonitrile, methanol, and methylene chloride, and ferrous and ferric chloride were carefully kept anhydrous. A buffer phosphate solution at pH 6.5 was used for some UV experiments.

Nitrates Degradative Reactions. All the reactions were conducted under nitrogen atmosphere and at thermostatted temperature (ranging between 37 and 60 °C). After the workup, the products were identified using standard techniques (NMR, GC−MS[a]) and compared with data reported in the literature. The formation of *S*-nitrosothiols was from two solutions containing the nitrate/thiol mixture and the iron(II) derivative and from reaction directly in a flat cell inside the spectrometer by a mixing flow system. The presence of the characteristic S−NO absorption band at $\lambda = 540$ nm ($\epsilon_{max} = 16.34\ M^{-1}\ cm^{-1}$) was followed. The reaction yield was more then 75% in comparison to the reacted nitrate for all the experiments. In particular, a cis/trans (90:10) mixture of the 5-phenyl-4-penten-1-nitrate (0.35 mmol) was dissolved in anhydrous CH_3CN or CH_2Cl_2, (4.0 mL) and was deoxygenated by bubbling of N_2 for 1 h. To this solution ferrous chloride (0.35 mmol) was added, and the solution was stirred at 60 °C for 60 min under N_2 atmosphere. The workup of the reaction allowed us to identify, besides the corresponding alcohol, the products **1** [GC−MS = (M^+) 106, 105, 77, 51, 28], **2** [GC−MS = (M^+) 182, 105, 77, 51, 28] and **3a** (H-derivative) [GC−MS = (M^+) 161, 91, 71]. When the experiment was conducted in the presence of a Br atom donor (CBr_3Cl or *N*-bromosuccinimide), **1**, **2**, **3b** (Br derivative) [GC−MS = (M^+) 240, 171, 160, 91, 71] and **4a** (Br derivative) [GC−MS = (M^+) 240, 160, 133, 117, 105, 91, 77] were detected. When a Cl atom donor (SO_2Cl_2) was used, **1** and **4b** (Cl derivative) [GC−MS (M^+) 196, 160, 131, 115, 105, 91, 77] were detected. When the 5-nitrate-1-pentene (0.15 mmol) was reacted with ferrous chloride (0.3 mmol) in anhydrous acetonitrile (5.0 mL), besides unreacted nitrate and the corresponding alcohol, the compound **5** [GC−MS = (M^+) 86, 71, 56] was detected.

When a cis/trans (90:10) mixture of the 5-phenyl-4-penten-1-nitrate (1.0 mmol), ferric chloride (1.6 mmol), and GSH (3.0 mmol) was dissolved in a 50:50 anhydrous acetonitrile/methanol solution (10.0 mL), carefully deoxygenated under N_2 atmosphere, and stirred at room temperature for 72, besides the corresponding alcohol and the oxidized glutathione, **1** and **6** [ES-MS 199 $[M + Na]^+$; GC−MS = (M+) 176, 148, 105,77, 71] were detected.

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Supporting Information Available: UV−vis spectra of iron(II) and iron(III) in the presence and in the absence of the nitrite anion. This material is available free of charge via the Internet at http://pubs.acs.org.

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[a] Abbreviations: GC−MS, gas chromatography−mass spectroscopy; ES-MS, electrospray mass spectrometry.

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(27) The possible metal mediated reaction with Fe^{2+}/NO^{2-}/RSH to form the corresponding *S*-nitrosothiol (see ref 28), even if it cannot be excluded, is not supported by my results or at least is difficult to invoke. In fact, preliminary tests conducted on the interaction between the nitrite anion and iron(II) as well as iron(III) in water showed identical UV spectra, allowing the conclusion that it was not possible to discriminate which type of interaction was taking place.
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