The nitrite anion: the key intermediate in alkyl nitrates degradative mechanism.

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Alky nitrates, *in vivo*, are metabolized to yield nitric oxide, and thiol groups are considered necessary cofactors. This statement is based on studies that underline how these species potentiate hemodynamic responsiveness to nitrates in patients with ischemic heart disease. However, the role of thiols might be mediated by the formation of corresponding S-nitrosothiols, and a redox process is responsible for the nitrates' degradation: an enzyme, probably the cytochrome P450, is involved *in vivo*. Here, we report evidence that, *in vitro*, no reaction between thiols and alkyl nitrates takes place, but that stronger reducing agents, such as iron (II) derivatives, are necessary: alkoxy radicals and the nitrite anion are the reaction intermediates. The latter, in slightly acidic conditions, for instance mimicking ischemic conditions, is shown to nitrosilate thiols to the corresponding S-nitrosothiols: the real NO suppliers. Therefore, the direct release of NO from nitrates is excluded. Finally, the *in vivo* role of thiols on *depletion* and *tolerance* is also accounted for.

Even if a central role of thiols on nitrates degradative reduction has always been invoked, no one has never reported a study on exactly what their role is: do they act as direct reducing agents or is a different reductant, for example an enzyme, responsible? The answer to this query was obtained when thiols such as glutathione (GSH), cysteine or benzylthiol, and nitrates such as the 5-phenyl-4-pentene-1-nitrate and the 1-penten-5-nitrate, in anhydrous solvent, were reacted: thiols and nitrates were recovered unchanged. That confirmed the non-involvement of thiols in the initial reductive

process, but indirectly led to hypothesize, *in vivo*, the predominance of the enzyme path: i.e., an Electron Transfer process between a reductant, the enzyme, and an oxidant, the nitrate.

In particular, the cytocrome P450, the enzyme most commonly involved in drugs metabolism,[1] is characterized by a heme group able to carry out electron transport by interconversion between Fe^{2+} (reduced) and Fe^{3+} (oxidized) states,[2] and therefore able to induce the reductive process. In fact, this enzyme, localized in the human heart and vessels, seems the most accredited, and its involvement in the biotransformation of organic nitrates, *via* a so-called two-electron mechanism, leading directly to the formation of NO, has been suggested.[3-6] However, this hypothesis contrasts with other reports[7-9] that highlight the intermediacy of S-nitrosothiols in NO release. Nevertheless, in principle, also *via* this two-electron degradative nitrate mechanism the formation of S-nitrosothiols could be accounted for, and that through the nitrosilation of the corresponding thiols. The latter process could be performed by the nitrous anhydride (N_2O_3),[10] possibly formed *via* oxidation of NO, but it has been shown to be irrelevant *in vivo* because negligibly slow.[11,12] Scheme 1.

In contrast, a mono-electron process, leading to alkoxy radicals and the nitrite anion, could more readily account for the formation of S-nitrosothiols.[8] In fact, the nitrite anion, *via* its acid equilibrium, leads directly to the formation of nitrosilating species. Scheme 1.

To support this hypothesis it was necessary to prove if the reduction of nitrates can really be induced by iron (II) derivatives and, to this end, nitrates such as the 5-phenyl-4-penten-1-nitrate and the 5-nitrate-1-penten should give clear evidence. In fact, the hypothesized alkoxy intermediates, besides forming the parent alcohols, can undergo rearrangements that lead to compounds whose formation is definitely a proof of a radical mechanism. Experiments conducted with these nitrates and anhydrous $FeCl_2$, in anhydrous solvents, led to the detection of derivatives such as **1**-**5**, Scheme 2.

The detection of these compounds is an unquestionable result in favor of a radical mechanism and of the role of iron (II) as a mono-electron transfer agent. Among the reaction products the corresponding di-sulphide and the nitrite anion were also evidenced. In particular, as reported in a previous paper,[12] the nitrite anion has been shown to be able to perform the nitrosilation of GSH, in a buffer solution, starting from

pH 6.86, which is a value consistent with ischemic conditions. Thus, this same nitrosilating process, here conducted *in vitro*, could be hypothesized to occur also *in vivo*, and the S-nitrosothiols, *via* a homolytic and/or a redox mechanism, act as NO releasers.[14] Thus, the NO supplementation *in vivo*, starting from organic nitrates, goes through nitrosilated species formed *via* the nitrite anion.

One of the most troublesome aspects of organic nitrate ester therapy is the fact that patients can become refractory to their effects, i.e., a repeated and prolonged administration results in the development of *tolerance*, characterized by a decrease in NO production, together with a decrease in tissue thiols level. As confirmed by the renewed responsiveness to nitrate therapy when the thiol groups are restored.[15]

In particular, it has been hypothesized that the *tolerance* is affected by enzyme-activity,[3] i.e., it increases with enzyme degradation[16] (decrease of enzyme reduced-form). Indirectly supporting this were the results of experiments in which a cell sample treated in advance for 48 hours[17] with sodium nitroprusside still showed response to glycerol trinitrate treatment, thus leading to the conclusion that only NO coming from an enzymatic mechanism induces *tolerance*. Actually, proof of the role of enzymes on *tolerance* is the time necessary to restore cell bioactivity (bioconversion) in respect to nitrates -- most probably the time required to endogenously re-establish the reducing capacity of P450. This hypothesis is supported by experiments in which the administration of reducing agents (antioxidants) such as vitamin C, vitamin E and sulphydryl derivatives,[18-22] even if in different percentage, allows the reducing properties of P450 to be restored in a shorter period of time. Inside cells, the possibility to re-convert P450-iron (III) into P450-iron (II) seems mainly restricted to reductants such as thiols, and cysteine and GSH -- the highest in concentration -- being the most accredited. To prove this, experiments with nitrates such as the 4-phenyl-1-nitrate and the 5-phenyl-4-penten-1-nitrate, and $FeCl_3$, both in the absence and in the presence of thiols, were conducted. Experiments carried out in the absence of thiols evidenced no interaction, but when repeated in the presence of thiols such as GSH, cysteine, or benzylthiol, the nitrate degradative reaction took place, Scheme 3. In particular, experiments with the 5-phenyl-4-penten-1-nitrate and $FeCl_3$, in the presence of GSH, showed the formation of **1** and **6**, Scheme 3. This result was mimicking that obtained by reacting the same nitrate directly with iron (II), thus confirming its involvement also in

this process, and its formation *via* the reduction of iron (III) by GSH. Further evidence was obtained running experiments at steady nitrate concentration, and varying the thiol/iron (III) molar ratio; the highest yield in nitrate degradation was obtained for a 2.5:1 molar ratio, i.e., conditions in which the conversion of iron (III) into iron (II) is favored. This result supports the role of thiols in delaying *tolerance*, *via* restoring the reduced form of the enzyme that is responsible in the first nitrate degradative step.

The positive effect of added thiols on nitrates hemodynamic is well known, but their action seems confined to the extracellular compartment.[23] In fact, through experiments with both GSH and N-acetyl cysteine, the possibility of enhancing the glycerol trinitrate degradation in whole blood, but not in red blood cells, had been reported;[24] i.e., showing an accelerating effect apparently mediated in the plasma fraction of whole blood. Thus, thiols supplementation is more likely related to a pronounced extracellular increase of these species, rather than intracellular. *(Plasma)* In particular, the extracellular hypertensive action seems related mainly to the presence of GSH that is synthesized intracellularly (mM concentration), starting from cysteine and, in normal conditions, continuously and irreversibly transferred out of the cell. Thus, the cysteine, definitely the main active intracellular thiol,[25,26] due to its manifold roles, i.e., reducing agent (in comparison to the enzyme), supplier of active S-nitroso cysteine and GSH producer, will diminish over time: this can accounts for *depletion*.

To explain the action of added thiols, because no direct interaction between nitrates and thiols can take place, in the extracellular compartment it is the presence of components such as ascorbate, urate and bilirubin, which can act as antioxidants, responsible for the degradative reduction of nitrates to nitrite. Only then will the supplemented thiols, under the indirect action of the nitrite anion be transformed into the corresponding S-nitrosothiols:[7] these will serve as *tolerance-reversing* mechanism.[27]

The degradative NO release from alkyl nitrates takes its start from the Electron Transfer process between the nitrate, the oxidant, and an iron (II) derivative, most probably the reducing enzyme P450, *in vivo*. This mechanism is supported by experiments that lead to the detection of products unquestionably supporting a radical mechanism, i.e., which can be accounted for only *via* an Electron Transfer process. The nitrate degradative process, besides alkoxy radicals, leads to the formation of the nitrite anion, in principle an inactive NO-derivative, but in slightly acidic conditions is able to

induce the nitrosilation of thiols to the corresponding S-nitrothiols: the real NO suppliers. Furthermore, because intracellular thiols, in particular cysteine and GSH, are involved in the fundamental processes inside the cell, their concentration will diminish over time: this accounts for both *depletion* and for the less efficiency in the re-establishment the enzyme reducing capability, *tolerance*.

Methods

Reagents. The 5-phenyl-4-penten-1-ol, synthesized as reported,[28] was reacted with N-Bromosuccinimide to lead to the 5-phenyl-4-penten-1-bromo, which under the action of silver nitrate guides to the 5-phenyl-4-penten-1-nitrate. The 5-nitrate-1-penten was obtained reacting the 5-Bromo-1-pentene, commercial grade, with silver nitrate. Gluthathione, cysteine, benzylthiol, ferrous and ferric chloride are commercial products; the latter two were carefully kept anhydrous. All solvents, acetonitrile, methanol and methylene chloride, were carefully kept anhydrous.

Nitrates degradative reactions. All the reactions were conducted in anhydrous solvents and under nitrogen atmosphere, at thermostated temperature (ranging between 37 and 60 °C). Bromotrichloromethane, or N-Bromosuccinimide, and SO_2Cl_2 were used as spin trap for carbon centered radicals. After the workup, products were identified using standard techniques (GC-MS), and compared with data reported in the literature. The formation of S-nitrosothiols was shown conducting experiments with two solutions containing respectively the nitrate and the iron (II) derivative, which were reacted directly in a flat cell inside the spectrometer by a mixing flow system. The characteristic S-NO absorption at 540 nm was detectable.

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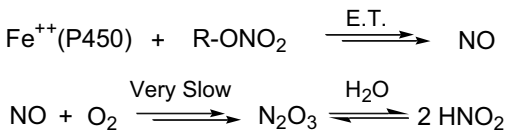
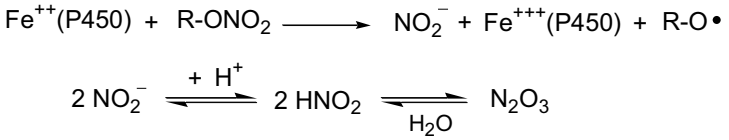
Scheme legends

Scheme 1: Nitrates degradation mechanism.

Two-electron mechanism

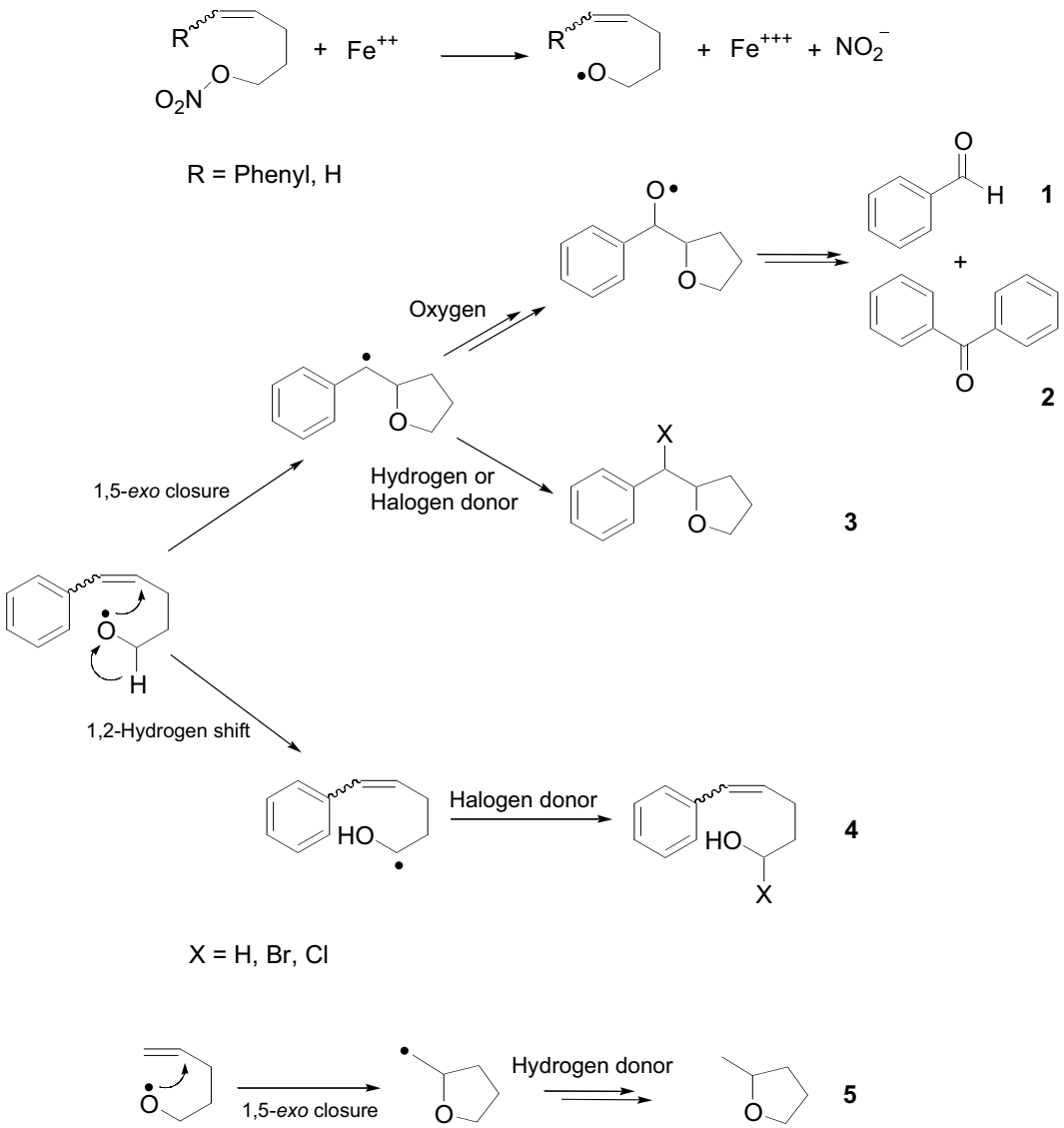


Mono-electron mechanism

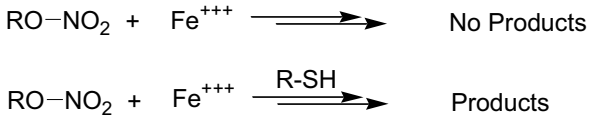


Nitrosilation

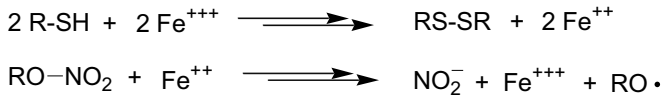
Scheme 2. Electron Transfer process: radical intermediates.



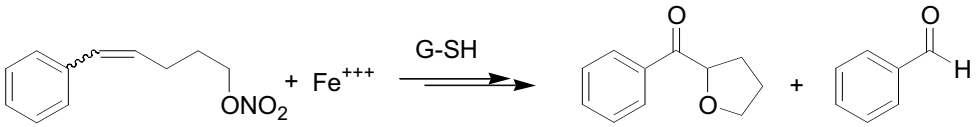
Scheme 3. The role of thiols in nitrates reduction.



The key step :



R-SH = GSH, Cysteine, Benzylthiol



6 **1**