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Article Addendum

Increased labile iron pool in sorghum embryonic axes after the exogenous application of nitric oxide is independent on the nature of the NO donor

Marcela Simontacchi, Sebastián Jasid and Susana Puntarulo*

Physical Chemistry-PRALIB; School of Pharmacy and Biochemistry; University of Buenos Aires; Buenos Aires, Argentina

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The objective of this work was to explore the hypothesis that nitric oxide (NO) affects Fe bioavailability in sorghum (*Sorghum bicolor* (L.) Moench) embryonic axes. NO content was assessed in embryonic axes isolated from seeds control or exposed to NO-donors, employing spin trapping electron paramagnetic resonance (EPR) methodology. NO donors such as sodium nitroprusside (SNP) and diethylenetriamine NONOate (DETA NONOate), released NO that permeated inside the axes increasing NO content. Under these conditions low temperature EPR was employed to study the labile iron pool. A 2.5 fold increase was observed in NO steady state concentration after 24 h of exposure to NO donors that was correlated to a 2 fold increase in the Fe labile pool, as compared to control axes. This observation provides experimental evidence for a potential role of NO in Fe homeostasis.

Nitric oxide (NO) has a wide range of functions, among them promotion of growth and seed germination were described in several plant species.[1] Evidences for its participation in Fe homeostasis in planta arise from the fact that Fe deficiency can be reverted enhancing NO level.[2] Moreover, it is expected that NO acts as intercellular messenger[3] being transported from the site of its synthesis. Nitrosylated Fe complexes, formed by reaction of NO with Fe^{2+} and biological thiols, have been proposed as NO carriers, since they are relative stable molecules.[4]

The ability of Fe of changing its oxidation state and redox potential in response to changes in the nature of the ligand makes this metal essential for almost all living organisms.[5] Fe-containing enzymes are the key components of many essential biological reactions. However, the same biochemical properties that make Fe beneficial might be a drawback in some particular conditions, when improperly shielded Fe can catalyze one-electron reductions of O_2 species that lead to the production of reactive free radicals. The toxicity of Fe depends on the Fenton reaction, which produces the hydroxyl radical (·OH) or an oxoiron compound ($LFeO^{2+}$) and on its reactions with lipid hydroperoxides.[6]

Most of the current information about NO functions in plants comes from pharmacological studies using NO donors, which generate NO either spontaneously, or after metabolic activation. Moreover, NO production from numerous compounds strongly depends on pH, temperature, light and the presence of reductants.[7] SNP and DETA NONOate have different kinetics and mechanisms of NO release. However, both are suitable compounds for long-term treatments, since their stability is higher than other NO donors.

In this work we evaluated NO steady state concentration in sorghum embryonic axes 24 h after imbibition, in control seeds (distilled water) and in seeds placed either in 1 mM SNP or DETA NONOate. SNP contains Fe in its chemical structure, thus a control was carried out employing photodegraded SNP, which consist of 1 mM SNP solution which had been left under light until all NO was released from the molecule. As it is shown in Table 1, NO released from the donors during the 24 h of treatment was incorporated to embryo tissues.

Imbibition of the seeds during 24 h in the presence of 1 mM SNP or DETA NONOate significantly increased fresh weight (FW) in axes, as compared to axes excised from seeds placed 24 h in distilled water (Table 1), suggesting a beneficial effect of NO upon the early stages of imbibition. When the seeds were incubated in the presence of photodegraded SNP, the FW remained in the control axes values, suggesting that NO released from SNP is responsible for the observed effect (Table 1).

In addition, in vivo exposure of sorghum seeds to NO donors protected axis membranes from the electrolyte leakage, which is understood as an indication of membrane injury due to oxidative damage (Table 1).

Labile Fe is defined as a pool of redox-active Fe composed by a weakly chelated Fe of both forms (ferrous and ferric) associated with a variety of ligands.[8] The labile Fe pool (LIP) was evaluated as the paramagnetic complexes formed by Fe and deferoxamine[9] (DF), that have a characteristic EPR signal at g = 4.3. Homogenates from

*Correspondence to: Susana Puntarulo; Physical Chemistry-PRALIB; School of Pharmacy and Biochemistry; University of Buenos Aires; Junín 956; Buenos Aires, Argentina; Fax: ++54.11.4508.3646; Email: susanap@ffyb.uba.ar

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sorghum embryonic axes mixed with 1 mM DF were examined by low temperature EPR and the formation of Fe-DF complexes was quantified. In this work we found that imbibition of seeds in the presence of NO donors led to an increase in the LIP assessed in homogenates of embryonic axes, as compared to control samples (Table 1).

The fraction LIP/total Fe in homogenates from embryonic axes was 2.1% for control axes and 5.2% after exposure of the axes to 1 mM SNP, suggesting that exogenous administration of NO could be related to an increase on Fe availability. The steady state concentration of the LIP could be understood as follows (eq 1), where each term refers to the change in the concentration of Fe bound to each physiological available Fe chelator in cells.

$$\frac{d[Fe]}{dt} = \left(\frac{d[Fe]}{dt}\right)_{citrate} + \left(\frac{d[Fe]}{dt}\right)_{ATP} + \left(\frac{d[Fe]}{dt}\right)_{ADP} + \left(\frac{d[Fe]}{dt}\right)_{oxalate} + \left(\frac{d[Fe]}{dt}\right)_{NO} + \left(\frac{d[Fe]}{dt}\right)_{other\ physiological\ chelators} \quad \text{(eq 1)}$$

NO could be bound to Fe and endogenous thiols generating dinitrosyl-Fe, dinitrosyl-diglutathionyl-Fe or dinitrosyl-glutathionyl Fe complexes among other nitrosyl-Fe complexes,[10] as indicated in (eq 2).

$$\left(\frac{d[Fe]}{dt}\right)_{NO} = \left(\frac{d[Fe]}{dt}\right)_{dinitrosyl\ complex} + \left(\frac{d[Fe]}{dt}\right)_{dinitrosyl\text{-}diglutathionyl\ complex} + \left(\frac{d[Fe]}{dt}\right)_{dinitrosyl\text{-}glutathionyl\ complex} + \left(\frac{d[Fe]}{dt}\right)_{other\ physiological\ complexes} \quad \text{(eq 2)}$$

After the exposure to 1 mM SNP even though total Fe content did not change (data not shown), LIP was significantly increased. This fact could be interpreted assuming that LIP was increased in the presence of supplemented NO by making Fe available in the cytosol (by allocation of Fe from other biological sources, such as ferritin) increasing the concentration of the nitrosyl-Fe complexes. These complexes have shown to be unable to induce oxidative stress in hepatocytes.[11] In this sense, in a chemical system NO inhibits the Fenton reaction by reacting with Fe (II) through the formation of nitrosylferrate (II) complex.[6]

In this work two different NO donors were able to increase FW of sorghum embryonic axes and showed a protective effect on membranes. On the other hand, it was found a direct relationship between NO steady state concentration and LIP levels in the axes. The formation of nitrosyl-Fe complexes may explain the beneficial effects of NO, in spite of the increased cellular LIP.

Table 1 Effect of NO donors on NO content and LIP in sorghum embryonic axes after 24 h of seed imbibition

	FW (mg axis^{-1})	Electrolyte leakage (%)	NO (nmol g^{-1} FW)	LIP (nmol g^{-1} FW)
Control	6.8 ± 0.3	29 ± 2	2.4 ± 0.2	8 ± 1
SNP	10.8 ± 0.6*	20 ± 1*	6.0 ± 0.9*	19 ± 2*
Photodegraded SNP	6.6 ± 0.3	27 ± 2	2.5 ± 0.6	9 ± 1
DETA NONOate	9.7 ± 0.9*	18 ± 1*	6.2 ± 0.6*	15.2 ± 0.5*

Sorghum seeds were exposed 24 h to distilled water (control) or 1 mM of the following chemicals: SNP, photodegraded SNP, and DETA NONOate. Axes were excised from seeds and employed for assays. NO content was determined by EPR in the presence of N-methyl-D-glucamine dithiocarbamate-Fe^{2+} as spin trap. LIP was estimated through the formation of DF-Fe (III) complexes in samples added with 1 mM DF and examined at low temperature EPR. *Significantly different from values for control embryonic axes at $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.,).

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