ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 3 & 4, 2005


# Original Research Communication

# Mechanisms of Vasodilatation Induced by Nitrite Instillation in Intestinal Lumen: Possible Role of Hemoglobin

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## ABSTRACT

**It has been shown that nitrite can be reduced to nitric oxide (NO) in intestine and a number of other tissues and released into the blood to form nitrosylhemoglobin (NO-Hb), existing in an equilibrium with *S*-nitrosohemoglobin. The latter has been suggested to be an NO transporter to distant organs. The aim of this study was to define the pathway of nitrite reduction to form NO in intestinal wall and to estimate whether this pathway has an effect on peripheral circulation. We have shown that in rat intestine at pH 7.0 70% of nitrite is converted to NO in mitochondria. At pH 6.0, nonenzymatic nitrite reduction becomes as efficient as the mitochondrial pathway. To prove whether the NO formed from nitrite in intestine can induce vasodilatation, sodium nitrite was instilled into intestinal lumen and the concentration of NO formed and diffused into the blood was followed by measuring of NO-Hb complex formation. We found that the concentration of NO-Hb gradually increases with the increase of nitrite concentration in intestinal lumen. However, it was not always accompanied by a decrease in systemic blood pressure. Blood pressure dropped down only after NO-Hb reached a threshold concentration of ~10 μ*M*. These data show that NO-Hb cannot provide enough NO for vasodilatation if the concentration of NO bound to Hb is < 10 μ*M*. The exact mechanism underlying vasodilatation observed when the concentration of NO-bound Hb was > 10 μ*M* is, however, not clear yet and requires further studies.** *Antioxid. Redox Signal.* 7, 515–521.

## INTRODUCTION

L-ARGININE VIA NITRIC OXIDE (NO) SYNTHASE is not the only source of NO occurring in the organism. Another source of NO is nitrite anion. Nitrite has been shown to be a source of NO *in vivo* (39). This phenomenon, the reduction of nitrite to NO, was observed under ischemic conditions when the oxygen levels required for the function of NO synthase are too low. Ischemia has been shown to stimulate NO synthase-independent NO generation in myocardium (38), skeletal muscles (25), and intestine (19). The following mechanisms were suggested to explain the release of NO from nitrite: comproportionation of two nitrite molecules (39), reduction of nitrite by low molecular reducing agents (35), by xanthine oxidoreductase (27), by deoxyhemoglobin (deoxyHb) (4, 28), and inside the cells by mitochondria and cytochrome P450 (17, 21). Recently, it has been shown that nitrite reduction to NO is the necessary step for NO release from nitroglycerin and probably other organic nitrates (1, 21). The mitochondrial pathway of nitrite reduction is completely inhibited by myxothiazol, the specific inhibitor of the $bc_1$ complex of the respiratory chain (17), indicating that complex III of the respiratory chain catalyzes this reaction. However, the balance between all these mechanisms has not been studied yet in a specific tissue. This is, however, important to understand which of those mechanisms is predominant under physiological and pathological conditions. Therefore, we looked at these mechanisms in intestine.

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NO formed in intestine during ischemia is released into the blood during reperfusion (18). In contrast to that, it has been shown that NO formed in blood cannot diffuse into the tissue due to high affinity to hemoglobin (Hb) (6, 18).

It has been suggested that NO should be present in the blood in nanomolar concentrations to fulfill its biological function (24). This suggestion is based on two important observations.

First, the $K_m$ for guanylate cyclase is in the nanomolar range of NO (13, 24); second, NO was measured directly with NO-selective quickly responding electrodes placed on the endothelial part of the vessels, performed by Malinski and co-authors (7). With other means, free NO was not detected in blood due to quick reactions with Hb. Measurements of the rates of these reactions show that the NO-mediated oxidation of oxyHb to methemoglobin yielding NOx [nitrate ($NO_3^-$ and nitrite ($NO_2^-$)] and the binding of NO to unoccupied hemes in Hb yielding nitrosylhemoglobin (NO-Hb) have very high rate constants, ($3.7 \times 10^7\ M^{-1}\ s^{-1}$ and $2.6 \times 10^7\ M^{-1}\ s^{-1}$, respectively) (10). These observations induced numerous studies of the interaction between free NO and Hb, a potent NO trap in the blood. It has been shown that disappearance of NO in the presence of Hb is very fast, and this fact created a doubt that free NO accounts for the actions of the endothelium-derived relaxing factor (26). NO-Hb, a product of the reaction between NO and Hb, does not possess significant biological activity due to the high stability of this complex. A significant development in the understanding of the interaction between NO and Hb and its biological role was done by Gow and co-authors (9, 10). They have shown that oxygen drives the conversion of NO-Hb in the "tense" T (or partially nitrosylated, deoxy) structure to *S*-nitrosohemoglobin (SNO-Hb) in the "relaxed" R (or ligand-bound, oxy) structure. In the absence of oxygen, nitroxyl anion ($NO^-$) was suggested to be liberated in a reaction producing methemoglobin. The yields of both SNO-Hb and methemoglobin were found to be dependent on the NO/Hb ratio. Therefore, SNO-Hb became the focus of further studies as a possible bioactive molecule. Due to the fact that *S*-nitrosothiols are potential NO donors, SNO-Hb was suggested to act as an endogenous NO donor and physiological regulator of blood pressure (37). This assumption was supported by a number of studies where vasodilatory activity of NO-Hb has been shown in perfused organs (3) and in aortic rings (29, 37). Later, however, Gladwin and co-authors have shown that SNO-Hb is not stable enough and releases nitrate (8), rather than turning back to NO-Hb. Moreover, recently, it has been shown that spectral changes suggestive of changes in levels of heme-bound NO (interpreted earlier as the transformation to SNO-Hb) are based on iron coordination shifts between hexacoordinate and pentacoordinate forms of NO-Hb rather than on redox- or oxygen-linked alterations of NO-Hb levels (5). It has been suggested that previous data outlining the significance of SNO-Hb formation and the equilibrium between NO-Hb and SNO-Hb were due to incorrect detection of SNO-Hb (5, 8). Therefore, NO-Hb is more likely the main reservoir of NO for blood, and the question of whether this complex can induce vasodilatation at reasonable concentrations is still open. In this study, we have addressed this question, studying the levels at which NO-Hb can occur in blood before systemic blood pressure is affected.

We have chosen intestine for this study, because intraluminal instillation of nitrite allows at least partial separation of nitrite and NO formed from nitrite and diffused in circulating blood. The concentration of NO-Hb complexes in blood is a good marker of the NO released in blood because it can be precisely measured by spin resonance spectroscopy (12, 14, 20, 36).

Therefore, the aim of this study was to clarify whether nitrite reduction in intestinal wall can induce systemic vasodilatation and to understand the underlying mechanisms involved in nitrite reduction and in delivery of NO to the distant tissues.

## MATERIALS AND METHODS

### *Experimental design of* in vitro *experiments*

Adult male Sprague–Dawley rats weighing 250–300 g (Animal Research Laboratories, Himberg, Austria) were used in this study. The animals were starved overnight. Immediately after decapitation, the liver, heart, and intestine were removed to a beaker with ice-cold sucrose buffer (0.25 *M* sucrose, 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% ethanol, pH 7.4), cut into small pieces, and washed with the same buffer to remove remaining blood. After drying with paper, the weight of tissue pieces was determined and the same buffer was added in a ratio of 1:6 (wt/vol) tissue/buffer. The tissues were homogenized using a Potter–Elvehjem homogenizer (VWR International GmbH, Vienna, Austria). The homogenate obtained was filtered through three layers of surgical gauze, divided into portions of 1 ml, and frozen in liquid nitrogen until it was used. Tissue homogenate was incubated in a buffer mixture containing 50% sucrose buffer and 50% phosphate buffer (154 m*M* KCl, 20 m*M* Tris, 4 m*M* $KH_2PO_4$, 1 m*M* EDTA, 2 m*M* $MgCl_2$, pH 7.35). Each sample contained 600 μ*M* Hb prepared from porcine erythrocytes as previously described (33). The samples were kept in a homemade chamber under a flow of nitrogen for 20 min on a shaking table to provide gentle mixing of samples with nitrogen. Afterwards, 10 μl of sodium nitrite ($NaNO_2$; final concentration 40 μ*M*) was injected into the samples under anaerobic conditions. The samples were aspirated in a syringe without exposure to oxygen and kept at 20 ± 2°C inside a homemade anaerobic chamber. After 40 min of incubation, the samples were frozen in liquid nitrogen for electron paramagnetic resonance (EPR) analysis.

### *Experimental design of* in vivo *experiments*

Adult male Sprague–Dawley rats weighing 250–300 g (Animal Research Laboratories) were anesthetized by intramuscular injection of a mixture of ketamine/xylazine (112/15 mg/kg body weight) and maintained under anesthesia by 0.2% isoflurane for the duration of the acute experiment. The animals were kept on a temperature-controlled surgical board (38 ± 1°C) and allowed to breathe spontaneously. The abdomen was opened, and the proximal intestine and distal ileum were identified and incised. Ringer solution supplemented with different nitrite concentrations was injected in jejunum. A silicon catheter was inserted in the femoral artery and used for measuring blood pressure and withdrawing blood samples. Mean arterial pressure (MAP) was determined using Cardiosys

equipment (Experimetria, Budapest, Hungary) connected to a computer.

### *EPR analysis*

Blood or tissue homogenates were placed into 1-ml syringes and frozen immediately in liquid nitrogen. Subsequently, the sample was pressed out of the syringe and moved into a fingertip liquid nitrogen Dewar for EPR analysis. EPR spectra were recorded at liquid nitrogen temperature with a Bruker EMX EPR spectrometer (general settings: microwave frequency, 9.431 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 4 G; gain, $10^5$). The double integrals of NO-Hb signals were calculated and compared with those obtained from nitroso-heme complex standards. The calibration of NO-Hb signals was performed as described previously (16) using aqueous solutions of NO, and using nitrite solutions reduced by dithionite as described by Tsuchiya *et al.* (34). Both calibrations gave identical results.

### *Presentation of results and statistical methods*

The data are expressed as means ± SEM, and the statistical significance of differences was estimated by the Mann–Whitney (*U*) test. The asymptotic significance level was set at 95% ($p < 0.05$).

## RESULTS

Intact intestinal homogenates show the typical EPR spectra containing very weak signals corresponding tentatively to semiquinones and non-heme iron proteins (Fig. 1A). The incubation of homogenates in the presence of nitrite and deoxyHb resulted in the appearance of an intensive signal typical for NO-Hb complexes (Fig. 1B). When the same incubation was made in the presence of myxothiazol, the intensity of the signal was significantly reduced (Fig. 1C). A small signal was observed when nitrite was incubated with deoxyHb without homogenate (Fig. 1D).

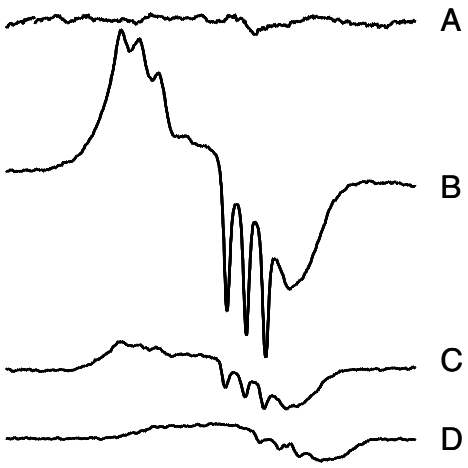


**FIG. 1. EPR spectra observed in intestinal homogenate (A), intestinal homogenate incubated with nitrite (B), intestinal homogenate incubated with nitrite and myxothiazol (C), and nitrite alone (D).** Each of the samples contained additionally 600 μ*M* deoxyHb used as NO trap.

Using myxothiazol, we compared the participation of the mitochondrial pathway to reduce nitrite to NO in liver, heart, and intestine. Figure 2 demonstrates that in both intestine and heart > 70% of nitrite reductase activity is myxothiazol-sensitive. In contrast, in liver only 40% of nitrite reductase activity is myxothiazol-sensitive, which is in agreement with previously published data (21). Thus, in both heart and intestine only 30% of nitrite reductase activity has nonmitochondrial origin. The nitrite reductase activity in heart was found to be higher than in liver and in intestine. We have chosen the intestine for further experiments because nitrite can be delivered to intestine through intestinal lumen without contact with the blood.

To understand better the role of the nonmitochondrial mechanism of nitrite reduction in intestinal wall, we studied the effect of pH on the reduction of nitrite to NO under anaerobic conditions in the following groups of samples: (a) buffer plus $NaNO_2$ alone to estimate the weight of the nonenzymatic mechanisms, (b) intestinal homogenate plus $NaNO_2$ plus 5 μg/ml myxothiazol to block nitrite reduction in mitochondria, and (c) intestinal homogenate plus $NaNO_2$ to estimate total nitrite reductase activity. As one can see, the mitochondrial mechanism dominated at physiological pH values. However, when the pH value was as low as 6, the other mechanisms became as efficient as the mitochondrial mechanism (Fig. 3).

Taking into account that nitrite can be reduced to NO in the intestinal wall, we performed further experiments with intraluminal instillation of nitrite expecting that NO formed in intestinal lumen would diffuse into the blood and form NO-Hb complexes, which then would deliver NO to other tissues. In fact, we observed that the concentration of NO-Hb in blood increased after nitrite instillation as a function of time and intraluminal nitrite concentration (Fig 4, inset).

However, the levels of NO-Hb were not directly correlated with a decrease in blood pressure. Only after NO-Hb concentration was higher than ~10 μ*M* we did observe a significant drop in blood pressure (Fig. 4).

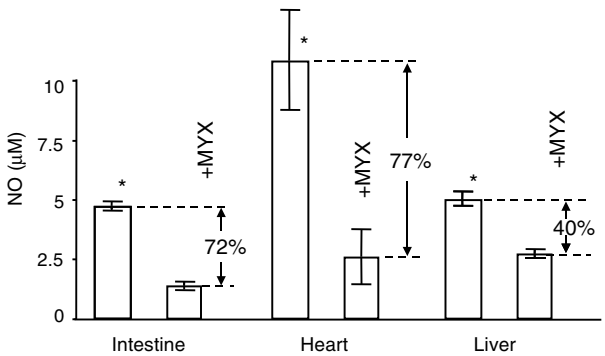


**FIG. 2. Effect of myxothiazol (MYX) on the nitrite reductase activity in homogenates prepared from intestine, heart, and liver.** *Significantly different from the myxothiazol-containing samples ($p < 0.006$).

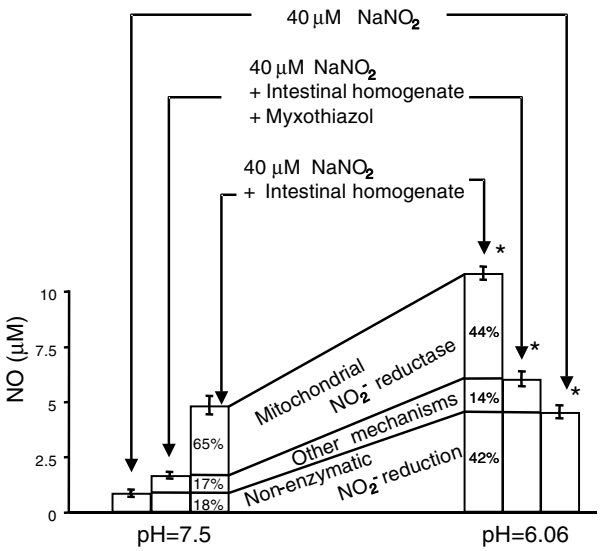


**FIG. 3. The dominance of different mechanisms generating NO from nitrite in intestinal homogenate at physiological and acid pH values.** *Significantly different from the corresponding value at pH 7.5 ($p < 0.006$).

## DISCUSSION

It has been reported that cytochrome P450 and $bc_1$ complex of liver mitochondria are the main reductants of nitrite to NO (17, 21). This has been shown by using determination of nitrite reductase activity and specific inhibitors in both homogenates and subcellular fractions. These studies have shown that myxothiazol is the most efficient inhibitor of mitochondrial nitrite reductase activity. Using this inhibitor, we have shown here that in rat intestine and heart the main part of nitrite reductase activity is located in the $bc_1$ complex of mitochondria. Additionally, we have shown that the nonenzymatic reduction of nitrite to NO, observed in the absence of homogenate, becomes as fast as the $bc_1$-mediated pathway at low pH values. Thus, in intestinal wall nitrite can be an important source of NO. To understand the relevance of this mechanism *in vivo,* we carried out the experiments with nitrite instillation in the intestinal lumen. These experiments demonstrated that the nitrite instillation in lumen results in the formation of NO-Hb complexes in blood and can induce systemic vasodilatation. Increased NO-Hb levels in blood did not necessarily result in a decrease in blood pressure. There was a threshold concentration of NO-Hb complexes after which we observed a decrease in systemic blood pressure. This shows clearly that if NO is bound to Hb at concentrations lower than 10 μ*M*, it is not enough to induce systemic vasodilatation. To define pathological circumstances that produce NO-Hb levels exceeding 10 μ*M*, we compared the data on NO levels in control rats and rats subjected to ischemia–reperfusion and sepsis. These levels were measured by the same method as in our previous studies (Table 1). Table 1 shows that the rats subjected to endotoxic shock produce amounts of NO-Hb that are high enough to induce systemic vasodilatation. Therefore, only under septic conditions one can expect that NO-Hb becomes a source of NO capable of inducing vasodilatation.

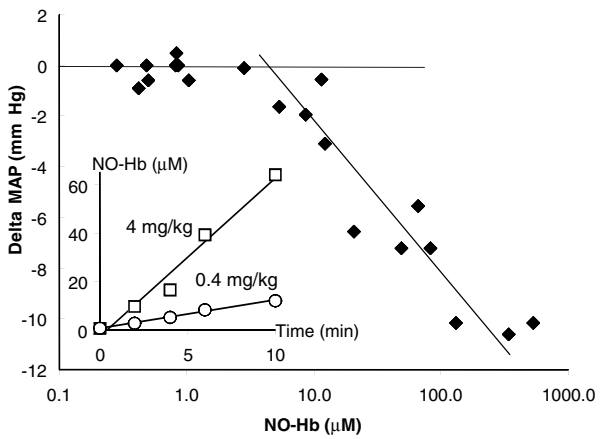


**FIG. 4. Effect of intraluminal nitrite instillation on the level of NO-Hb complexes in blood and MAP. (Inset)** The time course of NO-Hb complexes in blood after intraluminal nitrite instillation. Blood pressure was measured before blood sampling, and the plot of Delta MAP versus NO-Hb was made. Delta MAP is the difference between the values measured before and after nitrite instillation.

However, we do not have enough evidence to say that if NO-Hb levels are higher than 10 μ*M*, the vasodilatation is due to the release of NO from NO-Hb. This is due to the fact that under aerobic conditions NO is oxidized back to nitrite. In addition, a portion of nitrite can diffuse from intestinal lumen into the blood and can also be delivered to the tissues. It is rather impossible to separate these two mechanisms. As long as the concentration of NO-Hb was less than 10 μ*M*, no vasodilatation occurred in our model. This is clearly seen from our experimental data. As for NO-Hb concentrations above 10 μ*M*, the drop in MAP can be also due to the concomitant formation of nitrite from NO-Hb or other sources.

Therefore, our assumption is that at concentrations less than 10 μ*M* NO-Hb does not induce vasodilatation. This is clearly seen from our experimental data. However, the drop in MAP at concentrations of NO-Hb above 10 μ*M* can be due also to the concomitant formation of nitrite from NO-Hb or other sources.

The first one considers NO-Hb as the source of NO and that a small portion of NO remains unbound to Hb due to the thermodynamic equilibrium. When NO-Hb concentration is higher than the threshold of 10 μ*M*, then the concentration of

TABLE 1. THE CONCENTRATIONS OF NO-HB IN DIFFERENT EXPERIMENTAL MODELS

| *Experimental model* | *NO-Hb (μM)* | *Reference* |
|---|---|---|
| Laboratory rats | <0.100 | 19 |
| Sham | 0.193 ± 0.034 | 20 |
| Intestinal ischemia (1 h), reperfusion (0.5 h) | 0.536 ± 0.55 | 20 |
| Shock 6 h after LPS challenge | 20.4 ± 1.3 | 22 |

LPS, lipopolysaccharide.

free NO can reach the levels necessary for initiating vasorelaxation. It is known that those concentrations are in a range of nanomoles (13, 15, 24).

The second mechanism can be mediated by nitrite. In fact, there are a number of studies that showed a vasodilatory effect of nitrite infusions in humans (2, 11) and animals (23, 32). Therefore, the second mechanism would be nitrite-mediated vasodilatation. One can propose that nitrite is delivered to the tissues with the blood, and then diffuses into the tissues and releases NO by the mechanism described in this and previous studies (1, 21). NO released from nitrite induces local vasodilatation, but as nitrite is delivered to all tissues, this effect appears systemically.

Another mechanism of the vasodilatory effect of blood nitrite has recently been suggested by Cosby and co-workers (2). They suggested the reaction between Hb and nitrite to be the endogenous source of NO, which induces vasodilatation. The authors have shown that infusion of nitrite results in the consumption of nitrite from the blood, followed by the release of NO in the blood and induction of vasodilatation. All three events were facilitated by exercise. On the basis of these experiments, Cosby and co-authors proposed that nitrite is reduced by erythrocyte deoxyHb to NO and that NO formed in this reaction diffuses into the tissue and activates the guanylyl cyclase-inducing vasodilatation. Although the reaction of nitrite with deoxyHb yielding NO definitely takes place, our data suggest that this mechanism is not likely to operate *in vivo.*

This is based on the following arguments: (a) This reaction takes place under anaerobic conditions (4, 28). In the presence of oxygen, this reaction is shifted in favor of nitrate formation, as can also be seen from the data presented by Cosby *et al.* (2). Also, our data show that nonmitochondrial mechanisms including deoxyHb are relevant only at low pH. DeoxyHb at 600 μ*M* revealed very low nitrite reductase activity compared with that in mitochondria (see Fig. 1). (b) Even if we consider that NO has been produced in the reaction with deoxyHb, we face yet another problem. Both Fisch *et al.* (6) and our group (18) have shown that small portions of NO cannot diffuse from blood into tissue due to the high affinity to Hb. In contrast, NO formed in tissue diffuses into blood and forms NO-Hb complexes with erythrocyte Hb (18). In this study, we show that NO-Hb is probably able to release sufficient amounts of NO for vasodilatation, but only at extremely high concentrations (Fig. 4, Table 1). (c) Nitrite is able to diffuse not only into erythrocytes as was proposed by Cosby *et al.* (2), but also into tissue cells. In a tissue, nitrite releases NO at the $bc_1$ complex and activates guanylyl cyclase without further contact with the blood and consequently without being cached by Hb—a mechanism that we consider also to be important. (d) Cosby's study (2) did not explain the increased consumption of nitrite and the increased NO formation occurring during exercise. There is no reasonable explanation why exercise should increase the reduction of nitrite to NO by Hb. On the other hand, it is well known that exercise intensifies energy production in mitochondria (ATP synthesis) and stimulates endocytosis of plasma components (*e.g.*, glucose) into the cell to cover the energy deficit (30, 31). Endocytosis increases the uptake of specific molecules and also other small molecules like nitrite, which are unavoidably taken from the blood into the muscle cells. This favors the idea that NO is generated from the reduction of nitrite inside the cells and explains the increase in NO production during exercise.

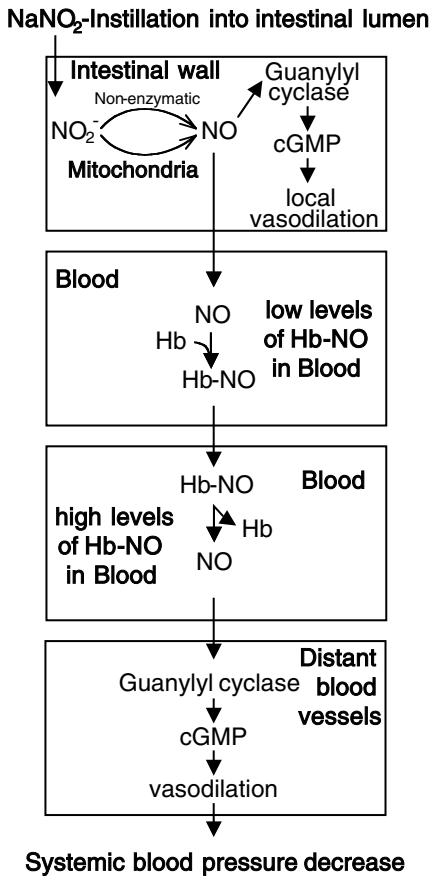


**FIG. 5. The proposed mechanism of the vasodilatation effect of intraluminal nitrite instillation.**

In summary, we propose that nitrite induces vasodilatation via intracellular reduction of nitrite in mitochondria to NO and activation of guanylyl cyclase without contact with the blood (Fig. 5). This mechanism operates independently of nitrite administration and is responsible for the local effects of NO. However, if the blood levels of NO bound to Hb exceed a certain threshold, NO-Hb probably becomes a source of NO sufficient to induce vasodilatation systemically (Fig. 5). The latter mechanism requires, however, further proofs.

## ABBREVIATIONS

deoxyHb, deoxyhemoglobin; EPR, electron paramagnetic resonance; Hb, hemoglobin; MAP, mean arterial pressure; NO, nitric oxide; NO-Hb, nitrosylhemoglobin; SNO-Hb, *S*-nitrosohemoglobin.

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Received for publication June 1, 2004; accepted October 4, 2004.

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