# The inhibitory effect of nitrite, a stable product of nitric oxide (NO) formation, on arginase

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**Abstract Macrophages contain arginase and an inducible NO synthase, demonstrated by using L-arginine, the common substrate, for production of both nitric oxide and urea. Arginase was inhibited by nitrite, the stable end product of NO. This inhibition was non-competitive, and could not be explained by the reaction of nitrite with arginine, or by the irreversible covalent modification of arginase, or by the removal of $Mn^{2+}$, a cofactor of arginase.**

*Key words:* Arginase; NO synthase; Nitrite; Inhibition; Fluorescence; Macrophage

## 1. Introduction

In macrophages, L-arginine is the substrate of both arginase (EC 3.5.3.1.) and NOS (EC 1.14.13.39.) [1–3]. The NO formed plays various roles in different cell types and is produced by various isoenzymes: in macrophages a $Ca^{2+}$-independent, cytokine-inducible enzyme has been observed [4]. This has been cloned recently [5] and is known to play an important role in reactions which are cytotoxic for tumors [6,7] and parasites [8]. Although its possible role in the survival of tumors has been described [9], the function of macrophage arginase has not yet been revealed. Nevertheless, a reciprocal induction of arginase and NO synthase synthesis regulated by Th-1 and Th-2 cytokines has been reported [10,11]. In addition, both enzymes are inhibited by the end product of the reaction that they catalyze: L-ornithine is a competitive inhibitor of arginase [12,13] and this can be explained based on their identical structural parts [13], while NO can block its own excess synthesis via binding to the porphyrin part of the active site of NO synthase [14,15]. The cross-regulation of the two pathways has not been studied in greater detail; however, we have observed that putrescine, a decarboxylated product of ornithine, decreases NO formation [13] markedly.

In this paper, we describe the inhibitory effect of nitrite, an oxidized derivative of NO, on arginase activity indicating another possible cross-regulation of the pathways utilizing L-arginine.

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*Abbreviations:* FBS, foetal bovine serum; DMEM, Dulbecco's minimal essential medium; IFNγ, interferon-γ; IL, interleukin; NOS, NO synthase; TLC, thin-layer chromatography; TNFα, tumor necrosis factor-α; amino acids are abbreviated by their usual three-letter symbols.

## 2. Materials and methods

### 2.1. Preparation of macrophages

CFLP mice (30–35 g) and Wistar rats (120–140 g) purchased from LATI (Gödöllő) were injected i.p. with 2% dephosphorylated casein as described earlier [16]. This treatment produces elicited macrophages. Peritoneal exudate cells were harvested after 96 h using $Ca^{2+}$-$Mg^{2+}$-free Hanks medium, centrifuged and then adhered to multiwell plastic plates (Corning) at a density of $10^6$ cells/well in 96-well plates and at $4\times10^6$ cells/well in 24-well plates, using complete Hanks medium at 37°C for 90 min. Adhered cells were used as macrophages. Based on previous studies [17], murine macrophages were used for arginase studies because of their high enzyme content while rat macrophages were tested for NOS showing greater activity compared to murine cells.

### 2.2. Purification and measurement of arginase

Arginase was partially purified from the supernatant of adhered murine cells by heat treatment at 60°C for 30 min followed by ammonium sulphate fractionation [18]. In numerous experiments commercial bovine liver arginase (Serva, Heidelberg) was used. Activity was measured on the basis of urea release over 30 min, urea being determined with diacetylmonoxime-thiosemicarbazide reagent using a spectrophotometric method [19].

### 2.3. Simultaneous measurement of arginase and NO synthase enzyme activities using labeled arginine

Arginase and NOS enzyme activities were directly measured in the lysate of casein-elicited murine macrophages by using [$^{14}$C]arginine as substrate and measuring the labeled ornithine and citrulline formed as product. $4\times10^6$ macrophages were cultured in 500 μl Arg-free DMEM containing 5% FBS and 1% Gordox in 24-well plates for 24 h, then lysed by 90 μl 5 mM HEPES (pH 7.4) containing 1 mM NADPH and then sodium nitrite in a 10 μl volume (final concentrations 1–10 mM) and 10 μl $^{14}$C-labeled Arg (spec. act. 11.3 GBq/mmol, Amersham, final Arg concentration adjusted to 100 μM using unlabeled Arg) were added and incubated at 37°C for 60 min. Reaction was stopped with 20 μl of a mixture of 5 mM unlabeled Arg, Orn and Cit for dilution of the labeled substrate and for visualization of the spots with 1% ninhydrin reagent on thin-layer chromatoplates. Arg, Orn and Cit were separated by TLC in a mixture of chloroform-methanol-ammonia-water (0.5:4.5:2:1) on silica gel plates (Merck) [20]. After visualization spots were cut out and counted for radioactivity in a Beckman LS 7800 spectrometer using a toluene-based scintillation cocktail containing Triton X-100 (2:1). Enzyme activity was calculated from the specific radioactivity of Arg, in pmol Orn and Cit per $10^6$ macrophages for arginase and NOS, respectively.

### 2.4. Kinetic studies on arginases

Kinetic studies were performed on partially purified macrophage arginase and commercially available bovine liver arginase. Since the $K_M$ of arginase is in the mM range [21], the mechanism was investigated in the presence of 0–50 mM Arg to determine the $K_M$ by constructing a Lineweaver-Burk plot, $K_I$ being determined in the presence of 1–20 mM $NaNO_2$ at 10 and 100 mM Arg by using a Dixon plot.

### 2.5. Measurement of urea as product of arginase action in intact cells

Adhered murine peritoneal macrophages were cultured in DMEM containing 10 mM Arg and 1–10 mM nitrite in an FBS-free DMEM for 24 h at 37°C under a 5% $CO_2$ atmosphere. Supernatants of these cultures were tested for urea production by a spectrophotometric method [19]. Cell-free samples were tested at each nitrite concentra-


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tion to obtain blank absorbance values for the correction of the color-forming reaction of nitrite and Arg.

*2.6. SDS-polyacrylamide gel electrophoresis*

The homogeneity of commercial arginase was assessed by performing SDS-PAGE analysis. A 10% slab gel was used for separation and the running buffer was diluted 10-fold from a stock solution containing 30.3 g Tris-HCl, 144 g glycine and 10 g SDS per l. Protein samples were treated at 100°C for 5 min in a buffer containing 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.025% bromophenol blue (final concentrations). Thereafter, running gels were stained using 1% Coomassie blue for 10 min and then washed thoroughly with 10% methanol-10% acetic acid prior to destaining.

## 3. Results and discussion

*3.1. Effect of nitrite on arginase activity*

Sodium nitrite led to strong inhibition of arginase activity as measured in both the macrophage and bovine liver enzymes. This inhibition was kinetically non-competitive: the $K_M$ and $K_I$ values for nitrite were 8.33 and 4.9 mM, respectively (Fig. 1). It was noted that nitrate, the completely oxidized end product of NO, is not inhibitory to arginase activity. Although liver and macrophage arginases are different isoenzymes [22], we found that macrophage and bovine liver

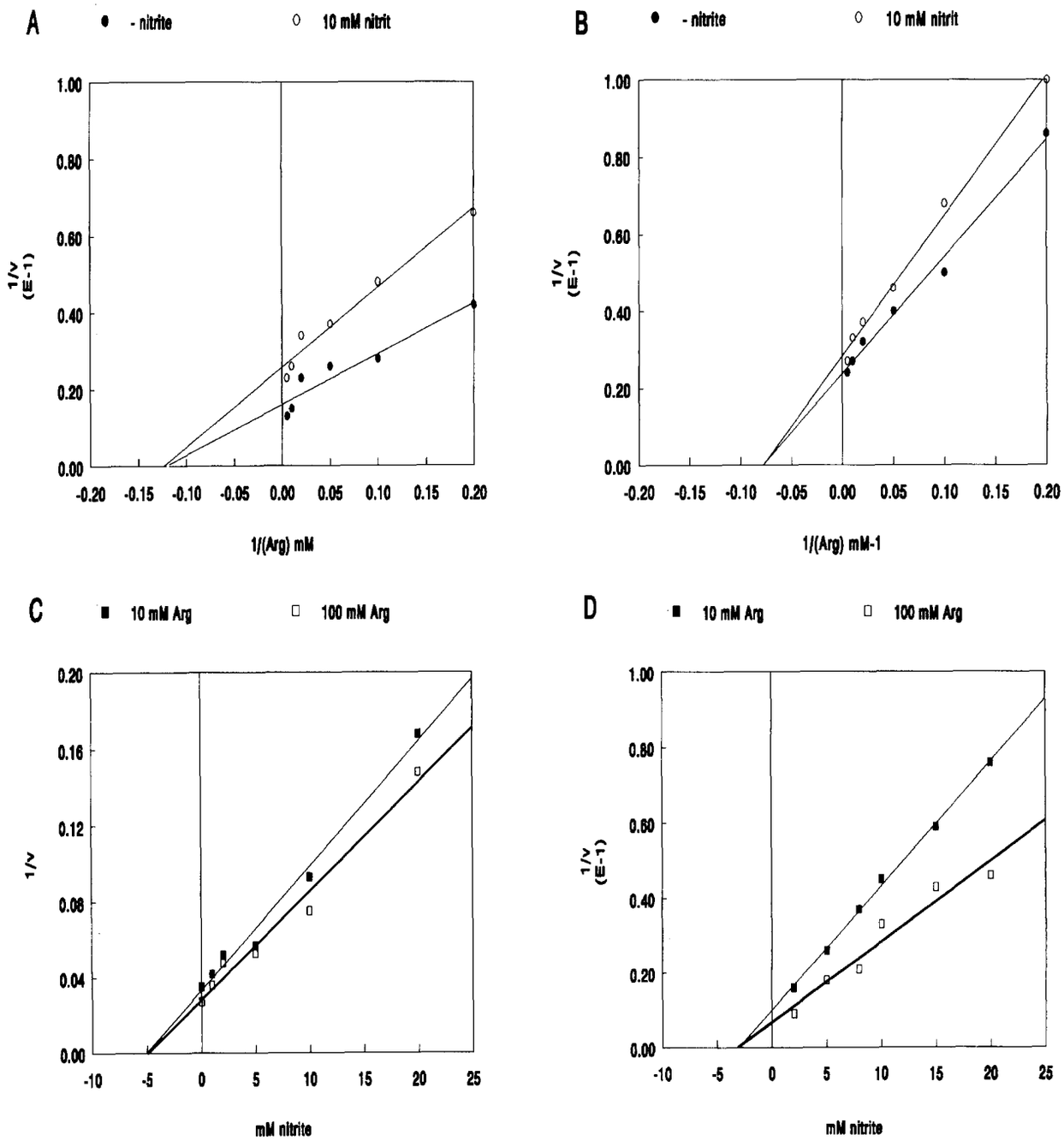


Fig. 1. Kinetic studies on arginase and NO synthase. Analysis of the inhibitory mechanism by Lineweaver-Burk plot in macrophage (A) and bovine liver (B) arginase. Both enzymes are inhibited via non-competitive mechanisms ($K_M$ 8.3 mM for macrophage and 12.5 mM for bovine liver arginase). Determination of the inhibitor constant ($K_I$) by Dixon plot in macrophage (C) and bovine liver (D) arginase ($K_I$ 4.9 mM for macrophage and 3.3 mM for bovine liver enzyme).

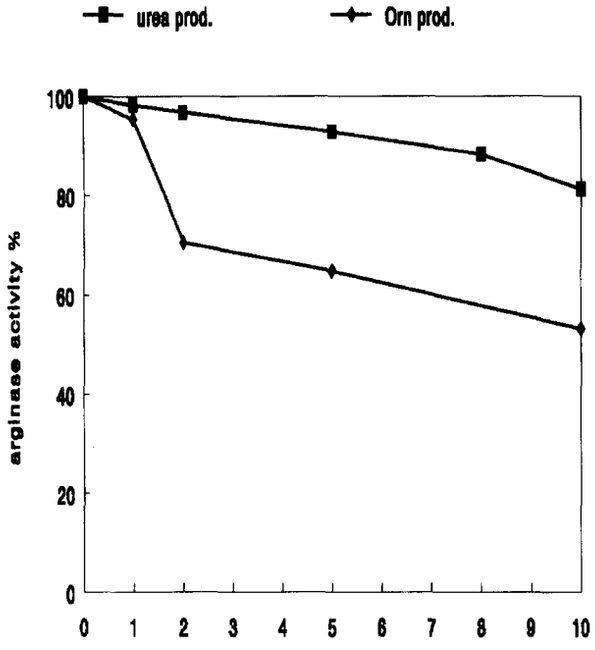


Fig. 2. Inhibitory effect of nitrite on arginase activity in intact and lysed macrophages. Urea production (■) was measured in the supernatant of intact macrophages after culturing for 24 h in FBS-free DMEM containing 10 mM Arg. The urea contents of all samples were corrected based on a cell-free supernatant to avoid interference from the reaction of arginine with nitrite (see Section 3.5). Ornithine production was measured at 0.1 mM $^{14}$C-labeled L-arginine concentration (♦). The product labeled Cit was separated by TLC and measured by liquid scintillation (described in Section 2.3).

arginase showed similar kinetic characteristics (Fig. 1). This is consistent with the observations of others [22,23] who have reported that arginase isoenzymes can be distinguished mainly by immunological methods.

*3.2. Effect of nitrite on urea production in intact cells*

Nitrite was also tested on urea production in murine peritoneal macrophages. Urea formation was found to be slightly inhibited (less than 20% even at 10 mM $NO_2^-$ in a 24 h culture in FBS-free DMEM (Fig. 2).

*3.3. Effect of nitrite on the transformation of labeled arginine*

1–10 mM sodium nitrite decreased the formation of labeled Orn from Arg, indicative of the inhibition of arginase in murine macrophages (Fig. 2). NO synthase could also be measured in this system but significant inhibition was not observed. Arginase was more markedly inhibited when measured directly, i.e. in cell lysates in the form of labeled Orn released from labeled Arg compared to the slight decrease of urea production in intact cells (Fig. 2). This discrepancy may be due to the probable limited entrance of exogenously added nitrite ions into macrophages where they could act on arginase. Another possible explanation may be the lower (suboptimal) labeled arginine concentration (0.1 mM; higher Arg concentration cannot be used because of the diluting effect of unlabeled Arg) used for the measurement of ornithine formation vs. 10 mM used in the measurement of urea production.

*3.4. Studies on the possible mechanism of the nitrite effect*

Our observations (data not shown) have suggested that nitrite reacts with arginine, yielding a urea-positive product showing the color reaction characteristic of ureido groups (e.g. citrulline is positive for this reason). We tested numerous compounds containing guanidino side chains. Although all tested compounds which had a free guanidino group (Arg, homoarginine, canavanine, γ-guanidinobutyrate, argininate) gave a slightly positive reaction, a significant contribution of this reaction to the inhibition of arginase is very unlikely due to the low concentration of the formed urea-positive compounds.

In another study, 50 μg/ml arginase was treated with 10 mM nitrite for 30 min at 37°C and then dialyzed against 0.01 M Tris-HCl pH 7.4-0.1 mM $Mn^{2+}$ in both the presence and absence of 10 mM $NaNO_2$. An untreated arginase sample was also dialyzed against the buffer without $NaNO_2$. Table 1 shows that nitrite-treated but buffer-dialyzed samples were not inhibited while samples dialyzed against the buffer containing nitrite were very strongly blocked. These studies suggest that the possible deamination reaction of free amino side chains of arginase with nitrite is not a likely explanation for the inhibitory effect of nitrite.

The inhibitory effect of nitrite cannot be abolished by raising the $Mn^{2+}$-concentration essential for arginase activity. Therefore, the effect of nitrite is not due to a possible reaction of nitrite with manganese.

*3.5. Possible biological significance of the arginase and NO synthase inhibition*

Nitrite ion is an end-product of NO in numerous cells – e.g. macrophages – where the Griess reaction is widely used to characterize NO production. Our results show that this stable product strongly inhibits arginase, another macrophage enzyme which utilizes arginine as substrate. These observations raise the possibility that the two arginine-utilizing pathways may cross-regulate each other because putrescine [13,24] and other polyamines [25,26] are inhibitory towards NO formation. However, the exact target point of these basic compounds has not been unambiguously demonstrated: spermine acts via its dialdehyde form on the induction of NO synthase [26] while putrescine has been observed as an inhibitor of NO synthase in macrophages ($K_i$=0.7 mM [13]) and in placenta cells [24]. The intermediate of NO formation, $N^G$-hydroxy-L-arginine is also an inhibitor of arginase [27]. Its physiological role is more established than that of nitrite because of the low $K_I$ value (42 μM). Although the nitrite concentration in macrophage supernatants (after 24 h) is below its effective inhibitory concentration on arginase, higher temporary nitrite concentrations cannot be excluded during NO synthesis. In the period when the bulk of NO is produced, a higher local nitrite concentration may play a role in the reversible inhibi-

Table 1
The activity of nitrite-treated and dialysed liver arginases

| Sample | Arginase activity |
|---|---|
| Control/control | 4208 ± 662 |
| Treated/control | 4279 ± 148 |
| Treated/treated | 1236 ± 84 |
| Non-dialyzed control | 4252 ± 684 |
| Non-dialyzed+nitrite | 876 ± 68 |
| Non-dialyzed+nitrite+$Mn^{2+}$ | 537 ± 101 |

Arginase activity is expressed in nmol urea/min per mg protein. Arginase concentration, 50 μg/ml; nitrite, 10 mM; $Mn^{2+}$, 0.1 mM. $n$=3.

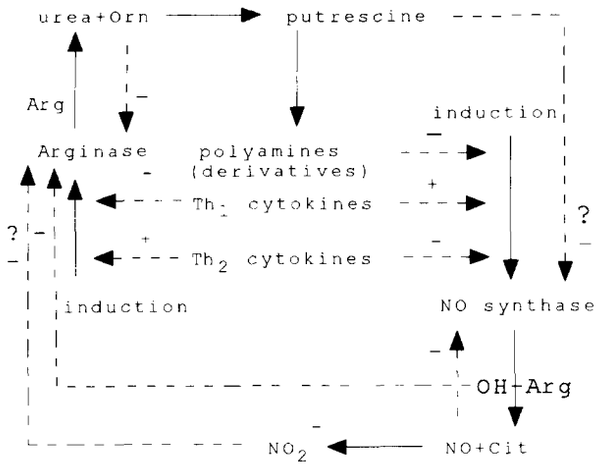


Fig. 3. Possible regulatory effect in arginine metabolism of macrophages. Th1 and Th2 cytokines regulate the induction of arginase and NO synthase reciprocally. Products of arginase and NO synthase (Orn and consequently polyamines, nitrite and $N^G$-hydroxy-L-arginine [OH-Arg], respectively) regulate reciprocally the activity (or induction) of the concurrent enzyme. Finally, Orn and NO cause end-product inhibition of the enzyme producing them (arginase and NO synthase, respectively). Metabolic routes are represented by continuous arrows, regulatory routes by dashed arrows. + and − denote activation and inhibition, respectively.

tion of arginase activity. Exogenous nitrite causes only slight inhibition of urea production in intact cells even at higher concentrations (see Section 3.2). This result can be explained by the limited transport of nitrite into macrophages. Moreover, our results showing the inhibition of arginase by nitrite were obtained at saturating substrate concentrations and this was not characteristic for the physiological intra- and extracellular (0.05–0.2 mM) space. The suboptimal physiological Arg concentration and the inhibitor-blocked arginase activity together are favorable conditions for NO synthase activity (its $K_M$ is in the μM order vs. the mM order for arginase). A more profound regulating effect is provided by cytokines, since Th-1 cytokines (IFNγ, TNFα, IL-1, IL-2) help the induction of NO synthase while Th-2 cytokines (IL-4, IL-10) favor the induction of arginase [14]. These possible regulating effects are summarized in Fig. 3.

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