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Arginase Activity is Inhibited by L-NAME, both *In Vitro* and *In Vivo*

DANIÈLE REISSER*, NATHALIE ONIER-CHERIX and JEAN-FRANÇOIS JEANNIN

Faculty of Medicine and Pharmacy, Cancer Immunotherapy Research Laboratory, Ecole Pratique des Hautes Etudes, Inserm U517, 7 Bd Jeanne d'Arc, 21079 Dijon, France

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The present study investigated the ability of the arginine analog L-NAME (N^{ω}-Nitro-L-arginine methyl ester) to modulate the activity of arginase. L-NAME inhibited the activity of arginase in lysates from rat colon cancer cells and liver. It also inhibited the arginase activity of tumor cells in culture. Furthermore, *in vivo* treatment of rats with L-NAME inhibited arginase activity in tumor nodules and liver, and the effect persisted after treatment ceased. The effect of L-NAME on arginase requires consideration when it is used *in vivo* in animal models with the aim of inhibiting endothelial NO-synthase, another enzyme using arginine as substrate.

Keywords: Arginase; L-NAME; NO-synthase

INTRODUCTION

Arginine is a substrate common to nitric oxide synthases and arginase. The highly soluble arginine analog L-NAME (N^{ω}-Nitro-L-arginine methyl ester) is an inhibitor of endothelial NO-synthase *in vivo*[1] and is often used in animal models for this purpose.

In mammals, the widely expressed enzyme, arginase (L-arginine amidinase; EC 3.5.3.1)[2] catalyses the hydrolysis of arginine to urea and ornithine. Ornithine is the precursor of polyamines that modulate proliferation and differentiation of both normal and tumor cells.[3] Arginase type I is mainly expressed in liver and arginase type II in other tissues. The effect of L-NAME on arginase had been studied *in vitro*,[4–5] but to our knowledge, its *in vivo* effect on arginase activity had not been considered.

The present study investigated the effect of L-NAME on arginase activity both *in vitro* and *in vivo*. L-NAME inhibited arginase expressed both in colon tumor cells and in liver. It also inhibited arginase activity in tumor cells in culture. Finally, *in vivo* treatment of rats with L-NAME inhibited the activity of arginase both in liver and in the experimental tumors induced by colon cancer cells.

MATERIALS AND METHODS

Cells and Peritoneal Carcinomatosis

The colon tumor cell line, PROb[6] was grown in Ham F10:Dulbecco media (3:1 v/v) (BioWhittaker, Fontenay sous Bois, France), fetal bovine serum 10% (Gibco BRL, Cergy Pontoise, France). The culture was regularly checked to be mycoplasma-free. Peritoneal carcinomatosis was induced by intraperitoneal injection of 10^6 viable syngeneic PROb cells in inbred BDIX rats, as previously described.[7] Typically, two weeks later, nodules of several millimeters in diameter develop in the peritoneal cavity of all the rats which usually die of their tumors between the 8th and the 12th week.

In Vitro Treatment with L-NAME

PROb cells were cultured for 24 h in the presence of various concentrations of L-NAME, hydrochloride, (Fluka, L'Isle d'Abeau-Chesnes, France) in 6 well plates at 37°C in a humidified atmosphere with 5% CO_2. Arginase activity was measured after washing the cell layer and detaching the cells with trypsin.

*Corresponding author. Tel./fax: +33-03-80-65-39-30. E-mail: reisserd@u-bourgogne.fr

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The toxicity of L-NAME on PROb cells, seeded in 96 well microplates, was evaluated by using the methylene blue assay.[8]

In Vivo Treatment with L-NAME

The effect of oral L-NAME treatment was tested, with a schedule used in studies aimed at inhibiting NO-synthase activity *in vivo*.[9]

Tumor-bearing rats were divided into two groups. In the treated group, L-NAME was added to drinking water (1.5 mg/ml = 200 mg/kg/day) from day 13 to day 33 after tumor cell injection. The control group did not receive any treatment. Rats were killed at day 21, 28 and 42 after tumor cell injection. Serum and liver samples, and tumor nodules taken from mesentery were frozen in liquid nitrogen and stored at −80°C until the evaluation of arginase activity.

Determination of Arginase Activity

Arginase activity was determined by measuring the urea produced from arginine hydrolysis as described by Modolell *et al.*[10] Briefly, tumor cells or tissues were lysed in PBS (phosphate-buffered saline) containing 0.2% Triton X-100 and 100 μg/ml of antipain, pepstatin and aprotinin (Boerhinger Mannheim, Meylan, France). After heating the lysate (50 μl) at 55°C in the presence of 5 mM $MnCl_2$ (50 μl) in PBS, 0.5 M L-arginine (Sigma, L'Isle d'Abeau-Chesnes, France) (100 μl) was added. The samples were incubated at 37°C for 1 h before stopping the reaction with the mixture H_2SO_4:H_3PO_4:H_2O (1:3:7 v/v). Samples were heated at 100°C for 45 min in the presence of α-isonitrosopropiophenone (Sigma). Urea concentration was measured at 540 nm. Results are expressed as units/10^6 cells or units/mg of protein (BioRad Protein Assay, BioRad, Ivry sur Seine, France) in tissues.

Statistical Analysis

Results are expressed as the mean (±SD) of determinations. The significance of differences between means was determined by paired Student's t-test in *in vitro* experiments, and by unpaired Student's t-test in *in vivo* experiments.

RESULTS

To study the effect of L-NAME on arginase both *in vitro* and *in vivo*, we chose PROb tumor cells, a clone of colon carcinoma origin, which produces peritoneal carcinomatosis in syngeneic rats, and liver which is the organ where arginase activity is the greatest.

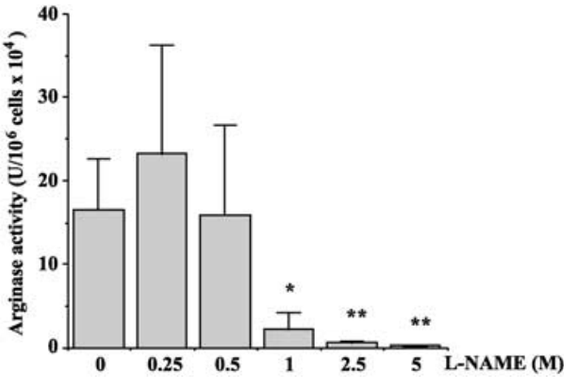

FIGURE 1 Effect of L-NAME on arginase activity in PROb lysates. Results are expressed as units arginase/10^6 cells × 10^4. Significance of the difference in activity with and without L-NAME is determined by paired t-test: *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$.

In Vitro Effect of L-NAME on Arginase Activity

Arginase activity is constitutive in PROb cells in culture (23 units × 10^{-4}/10^6 cells).

In a first set of 5 independent experiments, we used lysates of untreated cells and 100 μl of a mixture of 0.5 M arginine and various concentrations of L-NAME. In spite of L-NAME acidity, the pH remained close to 9.5, the optimum pH for arginase.[2] Arginase activity was significantly inhibited at a ratio of 1 M L-NAME/0.5 M arginine (Figure 1), showing a direct effect of L-NAME on arginase.

To see whether L-NAME effect was restricted to the arginase type present in these cells, we used rat liver lysates. In lysates, diluted to 20 μg total protein/ml, arginase activity was inhibited by 41 and 78% for L-NAME concentrations of respectively 2.5 and 5 M. Furthermore, L-NAME also inhibited the activity of commercial purified bovine liver arginase (Sigma) (data not shown). The inhibition was competitive with a Ki of 2 ± 0.16 mM, using an arginase concentration of 4 units/ml.

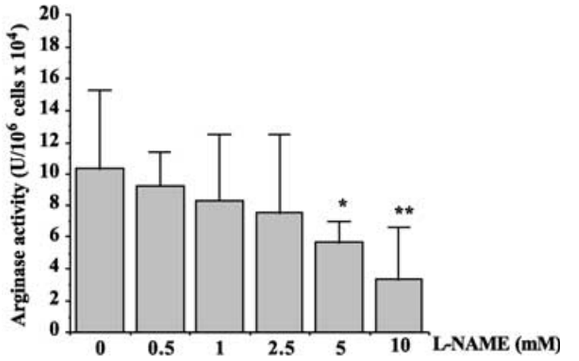

FIGURE 2 Effect of L-NAME on arginase activity in PROb cells. Results are expressed as units arginase/10^6 cells × 10^4. Significance of the difference in activity between control and treated cells is determined by paired t-test: *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$.

To determine whether of L-NAME had also an effect in living cells, we added L-NAME in culture medium. In a set of 6 independent experiments, incubation of PROb cells for 24 h with increasing concentrations of L-NAME reproducibly decreased arginase activity in a dose-dependent manner (Figure 2), showing that L-NAME was effectively transported into living cells. Inhibition was significant for an L-NAME concentration of 5 mM. As the culture medium contained 1 mM arginine, the efficient ratio L-NAME/arginine was 5. No toxicity of L-NAME was evident at any of the tested concentrations.

Ex Vivo Effect of L-NAME on Arginase Activity

In the animal model, we examined the effect of treatment with L-NAME on arginase activity both in liver and tumor tissue from the same rats. We first verified that the treatment did not induce life-threatening effects which could bias results. Urea concentrations were not different in sera from control and treated rats (data not shown); this reflected a normal regulation of urea in treated rats.

Arginase activity was significantly inhibited in the liver of treated animals (Figure 3), at days 28 and 42.

Arginase activities were always similar in different tumor nodules taken from the same rat, whatever their size. Nevertheless, the values given in Figure 3 are the means of the activities independently measured in 3 different nodules per rat. During L-NAME treatment, 21 and 28 days after tumor cell injection, arginase activity in tumors was lower in treated rats than in control rats, though the difference is not significant (Figure 3). At day 42, the *ex vivo* activity of arginase was significantly lower in tumor nodules from treated rats than in those from control rats.

In both liver and tumor nodules, arginase activity decreased at day 42, even though treatment had ceased for 9 days. The experiment could not be extended further, due to ethical considerations for the health status of control rats.

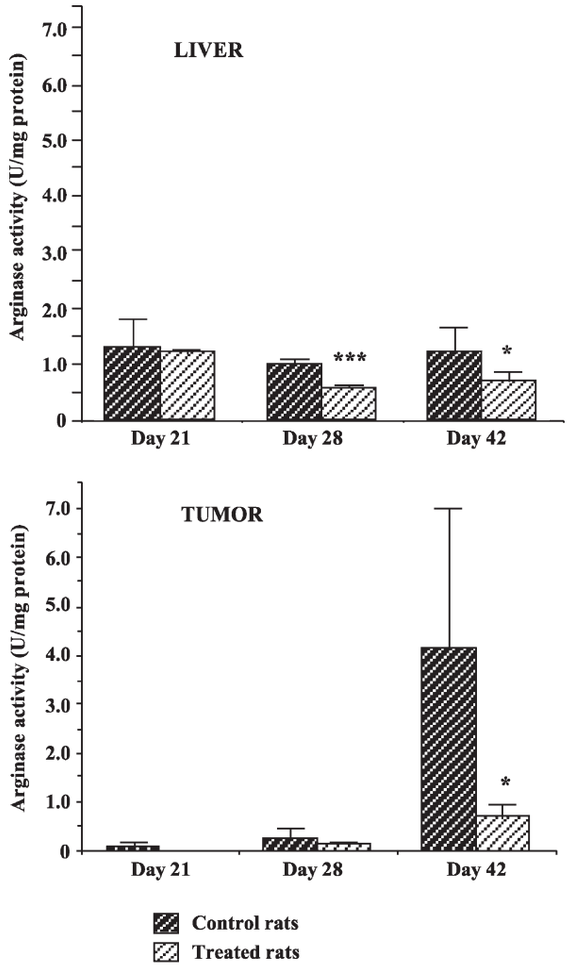

FIGURE 3 Effect of *in vivo* treatment with L-NAME on the *ex vivo* arginase activity in liver and tumors 21, 28 and 42 days after the injection of the tumor cells. Results are expressed as units/mg protein. Significance of the difference in activity between control and treated rats is determined by unpaired t-test: *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$.

DISCUSSION

Rat colon cancer cells PROb constitutively exhibited arginase activity in culture as was shown in human colon cancer cells.[11] This activity was inhibited by L-NAME both in broken cells and in living cells. The greater L-NAME/arginine ratio needed to inhibit arginase activity in culture may be due to the efficiencies of the uptake of L-NAME by cells, and of its conversion to its active metabolite, L-NNA (N^{ω}-Nitro-L-arginine).

L-NAME had not been found to inhibit arginase activity in two studies directly dealing with the subject. Hrabak *et al.*[5] used arginase from macrophages and liver, at 1/1 and 1/2 L-NAME/arginine ratios respectively, while we observed inhibition in a 2/1 ratio. Robertson *et al.*[4] showed that 140 μg rat liver arginase was not inhibited at a ratio of 20 mM L-NAME/20 μM arginine. The discrepancy with the present results may be due to the difference in liver arginase concentrations. However, the L-NAME metabolite, L-NNA had been shown to inhibit arginase type I[4] and, more recently, arginase type II.[12]

The arginase activity of PROb tumor cells was inhibited in the presence of L-NAME in culture medium for 24 h. Since cells were washed and the assay was performed in the presence of arginine in large excess by comparison of the L-NAME concentration in culture medium, arginase inhibition by L-NAME was not reversible. It is thus possible that L-NAME may act as a suicide substrate.

The effects of chronic L-NAME treatments on the development of experimental tumors are controversial as they have been shown to either inhibit,[13,14] or be without any effect[15] on tumor growth. These results have been considered in the light of NO-synthase inhibition, blood flow reduction in murine tumors,[16,17] as well as inhibition of tumor-induced angiogenesis,[18,19] but the effect of L-NAME on arginase activity in tumors and consequently the availability of ornithine was not studied. Indeed, treatment of tumor-bearing rats with L-NAME inhibited the arginase activity in tumor nodules, even at day 42 when this activity greatly increased in control rats. This increase might be linked with increased metabolism and polyamine synthesis in the tumor.

In vivo, inhibition was long-lasting, as evident 9 days after the end of treatment both in tumor and in liver. This excludes a simple competition for the active site of the enzyme, in accordance with *in vitro* results.

In conclusion, we describe for the first time the inhibitory effect of L-NAME on arginase *in vitro* and *in vivo*. L-NAME is generally used *in vivo* to inhibit endothelial NO-synthase. However, in these studies, its long-lasting effect on arginase activity should also be considered.

Acknowledgements

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