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Differential Inhibitory Effects of Three Nitric Oxide Donors on Ornithine Decarboxylase Activity in Human Colon Carcinoma Cells

François Blachier, David Briand, Mohamed Selamnia, Véronique Robert, Gwénaële Guihot and Camille Mayeur*

Laboratoire de Nutrition et Sécurité Alimentaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France

ABSTRACT. Ornithine decarboxylase (ODC, EC 4.1.1.17) is the enzyme responsible for the synthesis of polyamines, which are absolutely necessary for cell proliferation. In the present work, we tested the effects of 3 nitric oxide (NO) donors, namely, sodium nitroprusside (SNP), (Z)-1-(*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino] diazen-1-ium-1,2-diolate (MAHMA/NO) and 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium (DEA/NO), on ODC activity in human colon carcinoma cells (HT-29). SNP was the most effective inhibitor of ODC activity with a concentration of 8 μmol/L inducing 50% inhibition of basal activity. The effect of SNP was reversed by haemoglobin (Hb), but not by GSH or L-cysteine (CYS). Very little of the SNP in solution was degraded into nitrite, but the presence of cellular homogenate increased the production of nitrite. MAHMA/NO and DEA/NO were much less effective than SNP as ODC inhibitors, since the concentrations of these agents which induce 50% inhibition of basal activity were 20- to 60-fold higher than that of SNP. The effects of MAHMA/NO and DEA/NO were not reversed by haemoglobin. In solution, these latter 2 agents were totally degraded into nitrites. In conclusion, SNP on the one hand and MAHMA/NO and DEA/NO on the other appeared to release different NOx species with different efficiency on ODC activity. BIOCHEM PHARMACOL **55**;8: 1235–1239, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. NO donors; sodium nitroprusside; ornithine decarboxylase; colon carcinoma cells

Polyamines are strictly necessary for cellular proliferation [1]. Indeed, inhibition of polyamine synthesis in HT-29 human colon carcinoma cells leads to almost complete growth arrest [2]. Furthermore, decreased cell proliferation after confluency coincides with a spectacular fall in ODC† activity (EC 4.1.1.17) in these cells [3]. ODC is the enzyme responsible for polyamine synthesis. ODC catalyzes the production of putrescine from L-ornithine. This enzyme is considered a proto-oncogene central for the regulation of cell growth and transformation [4]. Although the exact mechanism of action of polyamines is not known, putrescine has been shown to stimulate DNA synthesis in intestinal cells [5] and to increase the transcription of growth-associated genes in human colon carcinoma cells [6]. L-arginine, apart from its role as an L-ornithine precursor for polyamine synthesis, is degraded into NO by NO synthase in HT-29 cells [3, 7]. NO is considered antiproliferative in several cell types [8–14], including HT-29 cells [15].

SNP is often used as an NO donor, but potential drawbacks are that SNP also releases cyanide and requires redox activation to produce NO [13]. In a recent study, it has been shown that $NO^{\cdot}$ rather than cyanide is the cytotoxic metabolite of SNP in isolated rat hepatocytes [16].

Thus, L-arginine is a precursor of 2 metabolites presenting opposite effects on cell proliferation. The aim of the present work was to characterize the effects of 3 NO donors on ODC activity in HT-29 cells.

MATERIAL AND METHODS

Reagents

L-[1-^{14}C] ornithine was purchased from Amersham. SNP, potassium ferricyanide, bovine haemoglobin, L-ornithine, reduced glutathione and L-cysteine were purchased from Sigma Chemicals. MAHMA/NO was purchased from Alexis Biochemicals. Diethylamine/NO complex or DEA/NO was purchased from Research Biochemicals International. Sodium nitrite was obtained from Aldrich. DFMO was kindly provided by Marion Merrell Dow Research Institute.

* Corresponding author: Dr. François Blachier, Laboratoire de Nutrition et Sécurité Alimentaire, bâtiment 230, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France. Tel. (33) 1 34 65 24 99, FAX (33) 1 34 65 23 11.

† *Abbreviations:* CYS, L-cysteine; DEA/NO, 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium; DFMO, D,L-α-difluoromethylornithine; DTT, dithiothreitol; Hb, haemoglobin; MAHMA/NO, (Z)-1-(*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino])diazen-1-ium-1,2-diolate; NO, nitric oxide; ODC, ornithine decarboxylase; SNP, sodium nitroprusside.

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HT-29 Cell Culture

HT-29 $Glc^{-/+}$ cells used in this study were cultured in standard Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 25 mmol/L of D-glucose, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 100 μg/mL of fungizone exactly as described [3]. HT-29 $Glc^{-/+}$ cells were used between passages P 53 and P 83 (1 passage every 7 days) and were seeded at a density of 0.40×10^6 cells per 25 cm^2 on day 0. The culture medium was changed every day, and cells were isolated at day 5–7 with EDTA-trypsin phosphate-buffered saline [3] and counted with an haemacytometer. The cells were washed, centrifuged, and the cell pellets kept at −80° up to the time of assay.

ODC Activity

ODC activity was measured using sonicated cells ($1.60 \pm 0.2 \times 10^6$ cells/assay) in a HEPES-NaOH (50 mmol/L, pH 7.2) buffer containing 200 μmol/L of pyridoxal 5-phosphate, 200 μmol/L of L-[1-^{14}C] ornithine, with or without 500 μmol/L of DTT and the tested agents. The assay mixture (100-μL final volume) was incubated for 60 min at 30°. The incubation was halted with 100 μL 0.5 mol/L of HCl, and the $^{14}CO_2$ was trapped in methylbenzethonium hydroxyde (Sigma) and counted by liquid scintillation. When tested in the presence of DTT, 10 mmol/L of DFMO inhibited ODC basal activity by 97 ± 3% ($N = 5$).

Nitrite and Protein Assays

Nitrite production from the NO donors was determined in the HEPES-NaOH (50 mmol/L, pH 7.2) buffer after 60 min at 30° using Griess reagent [17]. Production of nitrite from SNP by HT-29 homogenate was calculated by measuring nitrite production in the presence of SNP and by subtracting nitrite spontaneously released from SNP in the absence of homogenate and the endogenous nitrite content of the cell homogenate at the end of incubation, i.e. 2044 ± 388 pmol/mg of protein, $N = 3$. The amount of protein, as measured by the Lowry method [18], averaged 200 ± 15 μg per 10^6 cells ($N = 13$).

Statistical Analysis

Data are expressed as means ± SEM together with the number of individual experiments performed with HT-29 cells isolated at different passages (N). The statistical significance of differences between mean values was assessed by Student's *t*-test.

RESULTS

Inhibition of ODC Activity by SNP

In the first series of experiments, ODC activity was measured in the absence or presence of 500 μmol/L of DTT. The activity measured in the absence of DTT represented 65 ± 7% ($N = 4$) of the activity recorded in the presence of this reducing agent. In the subsequent experiments, ODC activity was measured in the absence of DTT, because this latter agent is known to react with ferricyanide.

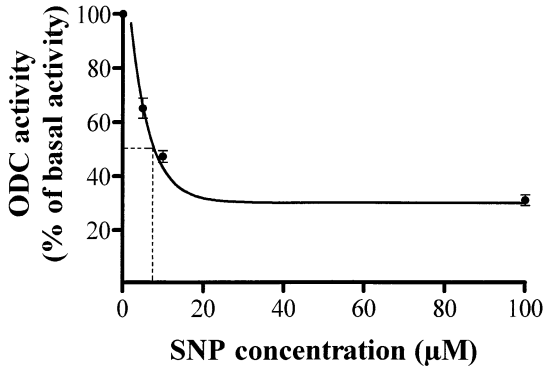

FIG. 1. Dose-dependent inhibition of ODC activity by SNP in HT-29 cell homogenate. The concentration of SNP which induced 50% inhibition of basal activity was found to be equal to 8 μmol/L. ODC activity was measured after 60 min incubation at 30° in the presence of 200 μmol/L of L-[1-^{14}C] ornithine and 200 μmol/L of pyridoxal 5-phosphate. ODC basal activity was 1725 ± 272 pmol/mg of protein/hr.

In HT-29 cell homogenate, ODC basal activity (i.e. 1725 ± 272 pmol/mg of protein per hr, $N = 15$) was dose-dependently inhibited by increasing concentrations of SNP after 60-min incubation (Fig. 1). The concentration of SNP which inhibited 50% of ODC basal activity was equal to 8 μmol/L. This inhibition was not mimicked by potassium ferricyanide, because this agent exerted no inhibiting effect on ODC activity (Table 1). Potassium cyanide was without effect at low concentrations, i.e. 5–10 μmol/L, but exerted a significant inhibitory effect on ODC activity at a higher concentration, i.e. 100 μmol/L. The ODC activity inhibition by 5 μmol/L of SNP was nearly abrogated by 30 μmol/L of Hb (Table 1). This Hb concentration reversed only partially, but significantly, the inhibitory effect of 100 μmol/L of SNP. The Hb concentration used in the present study was without effect on ODC activity. GSH (30 μmol/L) failed to reverse the inhibitory effect of SNP (Table 1). Even higher concentrations of GSH, i.e. 300 and 3000 μmol/L, failed to reverse the SNP effect (data not shown). Cysteine, another thiol-containing molecule, when used at 30 μmol/L, also failed to reverse the SNP effect. Three hundred to 3000 μmol/L of GSH or 30 μmol/L of cysteine were without effect on ODC activity (data not shown). Finally, 1 mmol/L of sodium nitrite exerted only a very weak inhibitory effect on ODC activity (Table 1).

Inhibition of ODC Activity by MAHMA/NO

We then tested another NO donor, namely MAHMA/NO. This agent also inhibited ODC activity in a dose-dependent manner (Fig. 2), but the concentration of MAHMA/NO which inhibited the basal activity by 50%,

TABLE 1. Effect of SNP and various agents on ODC activity in HT-29 cell homogenate

Tested Agents (μmol/L)	ODC Activity (% of basal)	
SNP (5)	62 ± 6 (6)	$P < 0.001$ vs basal
KCN (5)	96 ± 1 (3)	NS vs basal
SNP (5) + Hb (30)	89 ± 4 (3)	NS vs basal
Hb (30)	100 ± 12 (3)	NS vs basal
SNP (5) + GSH (30)	63 ± 3 (3)	NS vs SNP 5
SNP (5) + CYS (30)	63 ± 1 (3)	NS vs SNP 5
SNP (10)	47 ± 2 (3)	$P < 0.005$ vs basal
KCN (10)	97 ± 5 (3)	NS vs basal
SNP (100)	30 ± 2 (6)	$P < 0.001$ vs basal
KCN (100)	53 ± 4 (3)	$P < 0.01$ vs basal, $P < 0.001$ vs SNP 100
$K_3Fe(CN)_6$ (100)	96 ± 2 (3)	NS vs basal
SNP (100) + Hb (30)	48 ± 5 (4)	$P < 0.005$ vs SNP 100
SNP (100) + GSH (30)	28 ± 6 (4)	NS vs SNP 100
$NaNO_2$ (1000)	91 ± 2 (3)	NS vs basal

HT-29 cell homogenates were incubated for 1 hr at 30° in the presence of 200 μmol/L of L-[1-^{14}C]ornithine, 200 μmol/L of pyridoxal 5-phosphate, and the indicated agents. ODC basal activity was equal to 1725 ± 272 pmol/mg protein/hr. Results are means ± SEM together with the number of individual experiments (*N*). NS: not significantly different; $K_3Fe(CN)_6$: potassium ferricyanide; $NaNO_2$: sodium nitrite.

i.e. 153 μmol/L, indicates that this agent is less efficient than SNP for ODC activity inhibition. Both Hb and GSH were without effect on the ODC inhibition by MAHMA/NO (Table 2).

Inhibition of ODC Activity by DEA/NO

Another NO donor, namely DEA/NO, was tested for its effect on ODC activity (Fig. 3). This agent is able to inhibit ODC activity dose-dependently, with a concentration inducing 50% inhibition of basal activity equal to 507 μmol/L. This agent is therefore the least efficient NO donor tested for ODC activity inhibition. Again, both Hb and GSH were without detectable effect on the ODC inhibition by DEA/NO (Table 2).

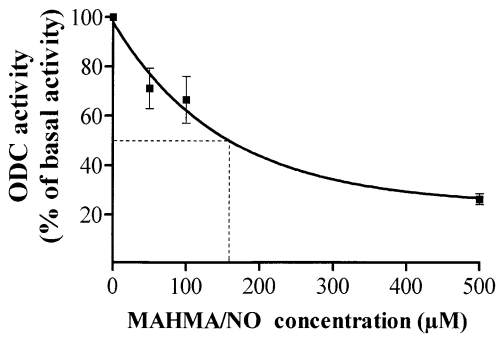

FIG. 2. Dose-dependent inhibition of ODC activity by MAHMA/NO in HT-29 cell homogenate. The concentration of MAHMA/NO which induced 50% inhibition of basal activity was found to be equal to 153 μmol/L. ODC activity was measured after 60 min incubation at 30° in the presence of 200 μmol/L of L-[1-^{14}C]ornithine and 200 μmol/L of pyridoxal 5-phosphate. ODC basal activity was 1725 ± 272 pmol/mg protein/hr.

TABLE 2. Effect of MAHMA/NO, DEA/NO and indicated agents on ODC activity in HT-29 cell homogenate

Tested Agents (μmol/L)	ODC Activity (% of basal)	
MAHMA/NO (500)	25 ± 3 (5)	$P < 0.001$ vs basal
MAHMA/NO (500) + Hb (30)	27 ± 1 (3)	NS vs MAHMA/NO 500
MAHMA/NO (500) + GSH (30)	26 ± 4 (3)	NS vs MAHMA/NO 500
DEA/NO (500)	45 ± 3 (3)	$P < 0.005$ vs basal
DEA/NO (500) + Hb (30)	44 ± 3 (3)	NS vs DEA/NO 500
DEA/NO (500) + GSH (30)	46 ± 4 (3)	NS vs DEA/NO 500

HT-29 cell homogenates were incubated for 1 hr at 30° in the presence of 200 μmol/L of L-[1-^{14}C]ornithine, 200 μmol/L of pyridoxal 5-phosphate, and the indicated agents. ODC basal activity was equal to 1725 ± 272 pmol/mg protein/hr. Results are means ± SEM together with the number of individual experiments (*N*). NS: not significantly different.

Nitrite Production from the Three NO Donors

The nitrite concentration measured from SNP in the incubation medium without HT-29 cellular homogenate was found to be very low (Table 3). In contrast, the same concentrations of MAHMA/NO and DEA/NO incubated under the same conditions for 60 min led to nitrite concentrations close to the concentration of the 2 NO donors (Table 3).

The production of nitrite from SNP was increased when 30 μmol/L of SNP (i.e. the concentration of SNP which gave the maximal inhibition of ODC activity, see Fig. 1) were incubated in the presence of HT-29 homogenate. Thus, after 60 min, a net production of nitrite by cell

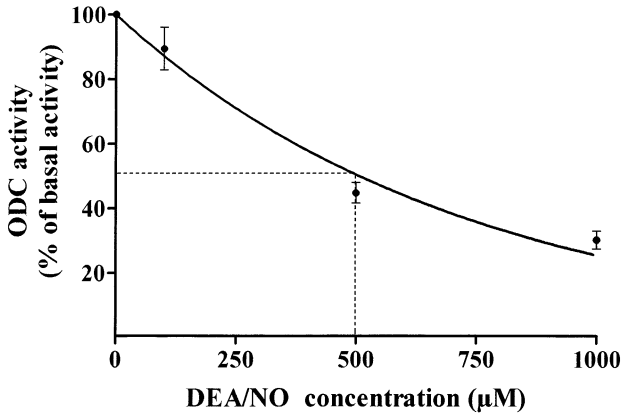

FIG. 3. Dose-dependent inhibition of ODC activity by DEA/NO in HT-29 cell homogenate. The concentration of DEA/NO which induced 50% inhibition of basal activity was found to be equal to 507 μmol/L. ODC activity was measured after 60 min incubation at 30° in the presence of 200 μmol/L of L-[1-^{14}C]ornithine and 200 μmol/L of pyridoxal 5-phosphate. ODC basal activity was 1725 ± 272 pmol/mg protein/hr.

TABLE 3. Nitrite production from the 3 NO donors

NO Donor (μmol/L)	Nitrite Production (μmol/L)
SNP (100)	0.9 ± 0.1
MAHMA/NO (100)	103.8 ± 12.6
DEA/NO (100)	89.5 ± 0.9

SNP, MAHMA/NO and DEA/NO were incubated in the absence of cell homogenate for 1 hour at 30° in a HEPES-NaOH buffer (pH 7.2) and nitrite concentrations were measured. Results are means ± SEM of quadruplicate experiments.

homogenate was calculated after correction for nitrite production from 30 μmol/L of SNP in the absence of cell homogenate and correction by endogenous nitrite present in cell homogenate. This homogenate-dependent nitrite production was found to be equal to 673 ± 174 pmol/mg protein/h, $N = 3$.

DISCUSSION

Among the 3 NO donors tested, SNP was the most effective inhibitor of ODC activity. In these experiments, DTT, which is often used as a reducing agent in the ODC activity assay [19], was omitted from the incubation medium, because this agent is known to react with ferricyanide [20].

The concentration of SNP which induced 50% inhibition of ODC activity in HT-29 cell homogenate was equal to 8 μmol/L and was thus 20- to 60-fold smaller than that for the other 2 NO donors tested, i.e. MAHMA/NO and DEA/NO. The experiment performed with potassium ferricyanide clearly demonstrates that this agent is without effect on this enzymatic activity and suggests that the effect of SNP was not due to the ferricyanide part of the molecule. Cyanide, which is released together with ferricyanide from SNP in solution [16], was without effect at low concentrations, i.e. 5 and 10 μmol/L. However, it clearly inhibited ODC activity at a higher concentration, i.e. 100 μmol/L. This result suggests that the inhibitory effect of SNP is due to NO release at low concentrations but that at higher concentrations, the release of cyanide from SNP likely plays a role in the inhibition of ODC activity. The spontaneous release of nitrite from 100 μmol/L of SNP in solution was low after 1 hr incubation, since it represented approximately 1% of SNP concentration (see Table 3). This nitrite production can, however, be increased in the presence of HT-29 homogenate. In our system, using 30 μmol/L of SNP (which produces maximal inhibition of ODC activity), we calculated that 10% of the added SNP is degraded into nitrite by the cellular homogenate after 1 hr incubation. Nitrite is considered as the end product of NO in aqueous solution [21, 22]. The compound(s) present in cell homogenate which is responsible for nitrite production from SNP is not known, but reductive metabolism of nitroprusside by rat liver microsomes leading to the production of NO in the presence of NADPH has already been demonstrated [23]. Furthermore, in our experiments, 30 μmol/L of haemoglobin was able to reverse either partially or totally—depending on the SNP concentration used—the inhibitory effect of SNP. Haemoglobin is used as an NO trap in several biological systems [24–26]. This result reinforces the view that SNP at 5–10 μmol/L inhibits ODC activity solely through the release of NO, but that at higher concentrations, cyanide together with NO inhibits enzymatic activity. In contrast, glutathione was without effect on the SNP inhibitory effect, even when used at high concentrations. Glutathione was used in this study because this molecule is able to react with NO to form S-nitrosoglutathione [27]. However, it should be noted that under certain conditions, S-nitrosothiols are able to release $NO^{\cdot}$ [28] and that nitroprusside is able to react with glutathione [29, 16]. Another thiol-containing molecule, i.e. cysteine, again failed to reverse the effect of SNP.

MAHMA/NO and DEA/NO when tested under the same experimental conditions were much less efficient than SNP as inhibitors of ODC activity, and the effect of these NO donors was not reversed by haemoglobin, suggesting that the NOy released from these compounds is probably different from the NOx released from SNP. Furthermore, as recorded in the presence of SNP, glutathione was without effect on the MAHMA/NO- or DEA/NO-induced ODC inhibition.

The spontaneous nitrite release from MAHMA/NO and DEA/NO in aqueous solution in the absence of cell homogenate was nearly total (see Table 3). The exact chemical nature of NO released from MAHMA/NO, DEA/NO or SNP is still controversial. Evidence has been presented in support of the production of $NO^{\cdot}$ from SNP [16, 30], while it has recently been claimed that SNP is a nitrosonium ion (NO^{+}) donor, which would be reduced by cysteine into $NO^{\cdot}$ [31].

In conclusion, in the presence of cellular homogenate, SNP releases significant amounts of NOx compound, which is trapped by haemoglobin. This NOx appears to be very efficient as an inhibitor of ODC activity. In contrast, MAHMA/NO and DEA/NO spontaneously release large amounts of an NOy compound which is not trapped by haemoglobin and which moderately inhibits ODC activity.

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