Journal of Chromatography, 539 (1991) 433–441
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 903

High-performance liquid chromatographic analysis of the unusual pathway of oxidation of L-arginine to citrulline and nitric oxide in mammalian cells

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ABSTRACT

A very unusual pathway of the oxidation of L-arginine to citrulline and nitric oxide has been discovered recently in cytotoxic macrophages. In an attempt to detect molecules generated through this metabolic pathway, a fast radio high-performance liquid chromatographic method was developed to analyse the whole set of radiolabelled L-arginine-derived metabolites produced by mammalian cells after appropriate induction. A new intermediate which might be N^G-hydroxy-L-arginine was found.

INTRODUCTION

It has recently been shown that mammalian cells can synthesize the highly reactive nitric oxide molecule ($NO^{\bullet}$), which was then identified as an important mediator in cellular communication [1]. $NO^{\bullet}$ biosynthesis has been demonstrated in, for example, cytotoxic macrophages [2] and stimulated endothelial cells [3]. The nitrogen atom of $NO^{\bullet}$ is derived from one of the two guanidino nitrogens of L-arginine via an unusual metabolic route. A proposed scheme, still speculative, is shown in Fig. 1. Citrulline and nitrite/nitrate, the oxidation products of $NO^{\bullet}$, are the stable products of the pathway [4]. In addition to macrophages and endothelial cells various cell lines, including the EMT6 [5] and TA3 murine adenocarcinomas [6], can generate these L-arginine-derived molecules after induction with appropriate cytokines such as interferon-γ [5]. The triggering of the $NO^{\bullet}$-generating pathway is associated with antiproliferative effects [5]. It has been involved, for instance, as an effector of the extracellular antiproliferative function exerted by cytotoxic macrophages against tumour cells [7,8].

The L-arginine: $NO^{\bullet}$ synthase activity responsible for the $NO^{\bullet}$-generating pathway has been found in 150 000 *g* cytosolic supernatants isolated from cytotoxic macrophages. It is activated by Mg^{2+} or Ca^{2+}, requires L-arginine, NADPH and tetrahydrobiopterin (BH_4) as cofactors [8,9] and is specifically inhibited by N^G-monomethyl-L-arginine (L-NMMA) [7,10,11] (Fig. 1).

In an attempt to detect molecules generated through this metabolic pathway and to investigate their role, we have developed a fast radio high-performance liquid

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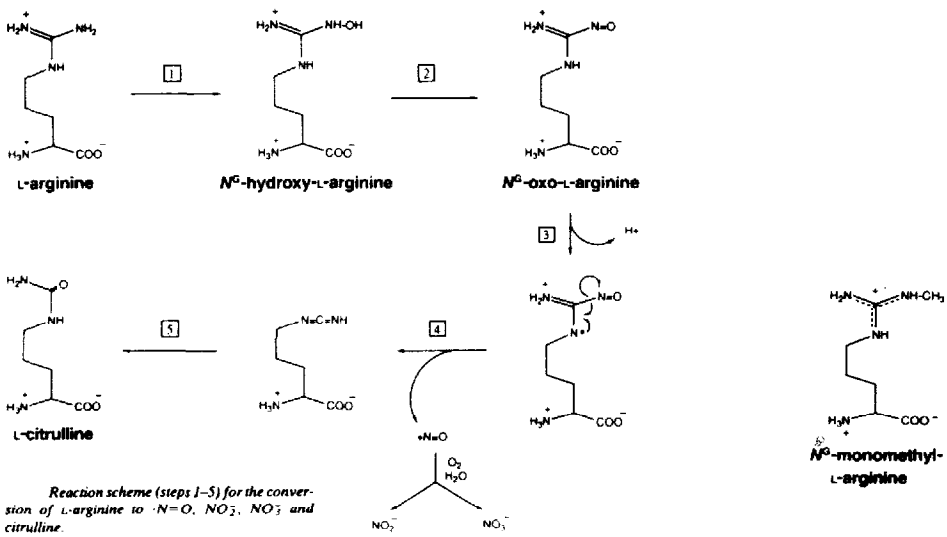

Fig. 1. Proposed scheme for the NO·-citrulline-generating pathway, according to Marletta [1].

chromatographic (HPLC) method to analyse the whole set of radiolabelled L-arginine-derived metabolites produced by suitable mammalian cells after appropriate induction.

EXPERIMENTAL

Chemicals and cytokines

N^G-Monomethyl-L-arginine (L-NMMA) was obtained from Calbiochem (La Jolla, CA, U.S.A.), 6-(*R,S*)-5,6,7,8-tetrahydro-L-biopterin from Serva (Heidelberg, Germany), L-arginine and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) from Merck (Darmstadt, Germany), dithioerythritol (DTE) from Sigma (St. Louis, MO, U.S.A.) and lipopolysaccharide (LPS) from *Salmonella enteritidis* from Difco (Detroit, MI, U.S.A.). L-[U-^{14}C]Arginine (11.1 GBq/mmol), L-[guanidino-^{14}C]arginine (1.85 GBq/mmol), DL-[1-^{14}C]ornithine (1.72 GBq/mmol) and [^{14}C]urea (2.2 GBq/mmol) were purhased from CEA (Saclay, France) and L-[ureido-^{14}C]citrulline (2.0 GBq/mmol) from NEN (Boston, MA, U.S.A.). Human recombinant tumour necrosis factor-alpha (TNF-α) and murine recombinant gamma interferon (IFN-γ), kindly provided by Dr. G. M. Adolf (Vienna, Austria), were from Gennentech (San Francisco, CA, U.S.A.). RPMI 1640 medium (containing 1.45 m*M* L-arginine) was obtained from Gibco (Cergy-Pontoise, France).

HPLC analysis of arginine metabolism

We chose a strong cation exchanger (sulphonate) linked to silica particles, *viz.*, a Zorbax 300 SCX column (250 × 4.6 mm I.D.) (Société Française Chromato Colonne, Neuilly Plaisance, France). The HPLC device included a Waters Assoc. Model 600 multi-solvent delivery system and the detector was a Berthold 506 C-1

radioactivity monitor connected to Epson PC AX microcomputer running under the Berthold HPLC program version 9.6. The following elution conditons were used: flow-rate, 1.0 ml/min; 0–7 min, isocratic, 0.02 *M* sodium citrate (pH 2.2); 7–17 min, linear gradient to 0.1 *M* sodium citrate (pH 3.0); 17–27 min, linear gradient to 0.2 *M* sodium citrate (pH 3.0); 27–35 min, isocratic, 0.2 *M* sodium citrate (pH 3.0).

Cell lines

The hydroxyurea-resistant TA3 M2 clone was obtained as described previously [12]. The EMT6 cell line is a murine mammary adenocarcinoma from BALB/c mice, kindly provided by Dr. G. Lopez-Berenstein (Tumor Institute, Houston, TX, U.S.A.).

TA3 M2 cytosolic extracts

These were prepared as described previously [6]. Briefly, TA3 M2 cells were treated for 24 h with appropriate additives. Lysates were centrifuged for 20 min at 4°C in a Beckman TL-100.2 rotor at 150.000 *g*. The supernatant was rapidly frozen in liquid nitrogen and stored at −80°C.

Determination of L-*Arginine: NO˙ synthase activity*

The following reagents were incubated in a final volume of 90 μl: 100 m*M* HEPES (pH 7.6), 15 m*M* magnesium acetate, 10 m*M* DTE, 130 μ*M* NADPH, 500 μ*M* BH_4, 8 kBq L-[guanidino-^{14}C]arginine (1.85 GBq/mmol) and the indicated concentrations of L-arginine to which appropriate amounts of cell extract were added. Where indicated, L-NMMA was used at a final concentration of 2.2 m*M*. Samples were incubated for 50 min at 30°C and then heated at 90°C for 2 min. Precipitates were pelleted and 200 μl of 0.02 *M* sodium citrate (pH 2.2) were added to the supernatant, which was filtered through a YM-10 ultrafiltration membrane (M_r cut-off 10000; Amicon, Danvers, MA, U.S.A.). A 50-μl aliquot of the filtrate was loaded onto the analytical column.

Analysis of EMT6 culture medium

EMT6 cell monolayers were obtained as described previously [5]. The medium was washed out and replaced with 1 ml of fresh medium RPMI 1640 supplemented with IFN-γ and LPS if the cells were to be induced. In some experiments, L-NMMA (2.2 m*M*) was added. Radiolabelled precursors were then added: 100 kBq of L-[U^{14}C]arginine (11.1 GBq/mmol) or 80 kBq of L-[guanidino-^{14}C]arginine (1.85 kBq/mmol) or 37 kBq of L-[ureido-^{14}C]citrulline (2.0 GBq/mmol). Supernatants were collected after 18 h and kept frozen at −80°C until anaysis. They were diluted with the starting elution buffer and processed in the same way as TA3 M2 cytosilic extracts: 50–100-μl samples were injected.

RESULTS

The low pH of the buffers (2.2–3.0) was chosen taking into account the p*K* values of the α-carboxyl groups of amino acids to control partly their ionization state (ornithine 2.0, arginine 2,2 citrulline 2.4). Typical retention times for authentic markers were urea 3.8 min, citrulline 11.5 min, ornithine 21 min and arginine 31 min (Fig. 2).

basic than ornithine and less so than arginine. It may be assumed that N^G-hydroxyarginine or N^G-oxoarginine would be less basic than arginine because of the loss of resonance energy which stabilizes the guanidinium cation. Thus, taking into account the chromatographic properties of X and the location of its radiolabelling (guanidino-^{14}C), it may be hypothesized that X is probably either N^G-hydroxyarginine or N^G-oxoarginine, the intermediates which have been postulated (Fig. 1). We are currently trying to obtain large amounts of the compound to check this hypothesis by mass spectrometry.

The metabolites of the NO$^\bullet$-generating pathway (NO$^\bullet$, N^G-hydroxyarginine) might be direct inhibitors of a key enzyme of DNA synthesis ribonucleotide reductase. This would explain the antiproliferative effects obtained through the induction of the NO$^\bullet$-generating pathway [6].

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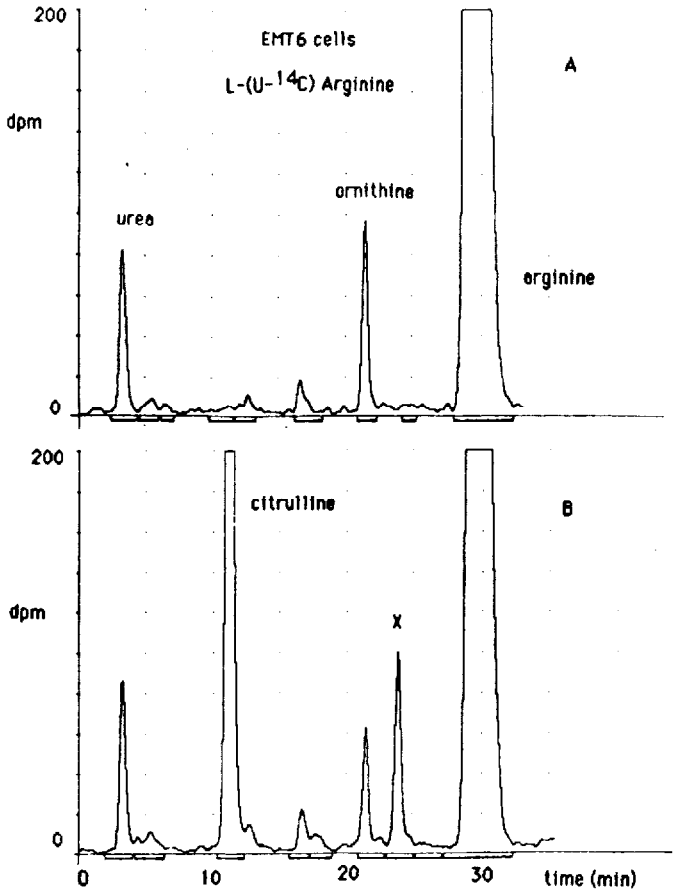

Fig. 3. HPLC analysis of untreated and induced EMT6 cell culture media. L-[U-^{14}C]Arginine: 100 kBq/ml. (A) EMT6 monolayers in 35-mm dishes (Nunclon) were incubated in 1 ml of RPMI 1640 medium for 18 h. (B) induced EMT6 cells were incubated for 18 h in 1 ml of RPMI 1640 supplemented with IFN-γ (40 U/ml) and LPS 100 ng/ml).

the concentration of arginine produced than to that of added citrulline. Moreover, the production of X and not that of arginine was again inhibited by L-NMMA (Fig. 6). It must be pointed out that X was not found in cell lysates. This metabolite was also demonstrated in cytosols of induced TA3 M2 cells supplemented with cofactors of the NO$^{\bullet}$-generating pathway (BH4, NADPH).

The NO$^{\bullet}$-generating pathway was induced in TA3 M2 cells by incubation for 24 h with IFN-γ (40 U/ml), TNF-α (150 U/ml) and LPS (10 μg/ml). When cytosols of induced cells were supplemented with BH_4 and NADPH, in the presence of L-[guanidino-^{14}C]arginine, the NO$^{\bullet}$-generating pathway was triggered and citrulline and metabolite X were generated. They were not detected in induced cells in the absence of cofactors or in non-induced cytosols even in the presence of cofactors. The production of citrulline and of metabolite X by induced cytosols was also here

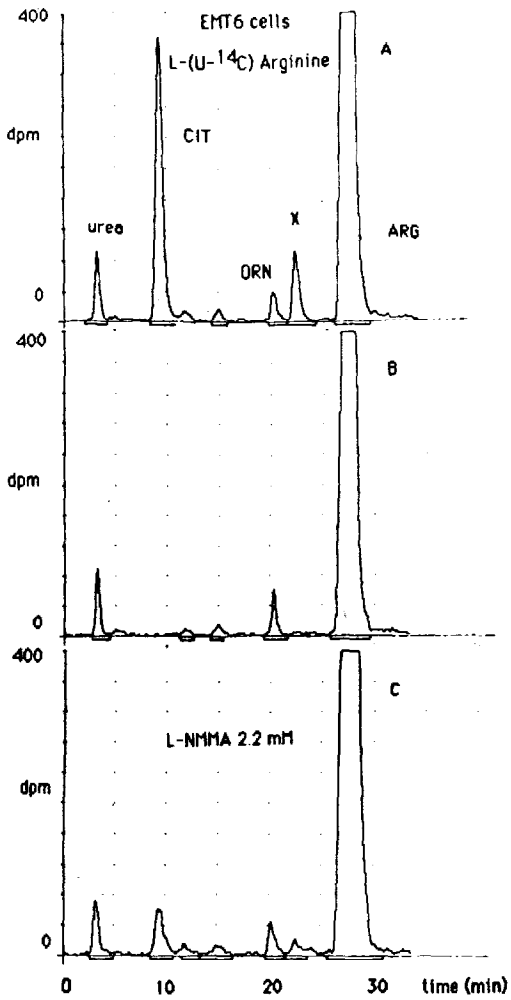

Fig. 4. Effect of L-NMMA on the production of L-arginine-derived metabolites by induced EMT6 cells in the culture medium. Cells were cultivated as described in Fig. 3. (A) EMT6-induced cells; (B) untrated EMT6 cells; (C) EMT6-induced cells cultivated in the presence of 2.2 m*M* L-NMMA.

inhibited by L-NMMA. The generation of the metabolite referred to as "creatine derivative" was also found in cytosols of TA3 M2 cells to the same extent regardless of whether the cells had been induced or not; this was not inhibited by L-NMMA (Fig. 7). The dependence of the formation of citrulline and X on arginine concentration was studied and we again found a nearly constant [X]/[citrulline] concentration ratio, which was close to 0.2. We failed to observe compound X in either lysates or supernatants of cytotoxic macrophages, although we could easily detect the production of citrulline.

The metabolite X was not very stable even when kept frozen at −80°C. It was

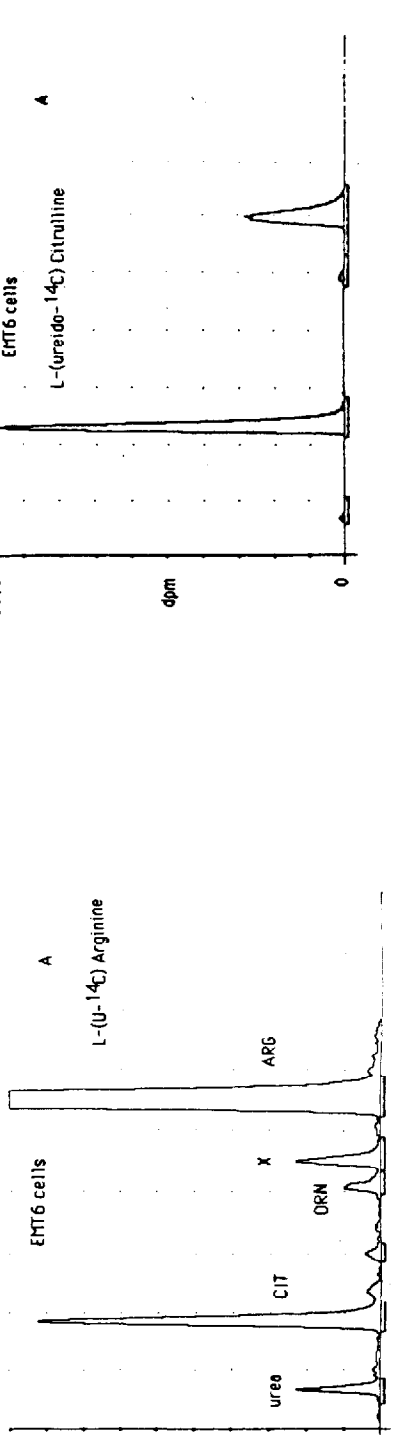

Fig. 5. Comparison of radiolabelled L-arginine-derived metabolites generated in induced EMT6 cell culture media in the presence of (A) L-[U^{14}C]arginine or (B) L-[guanidino-^{14}C]arginine.

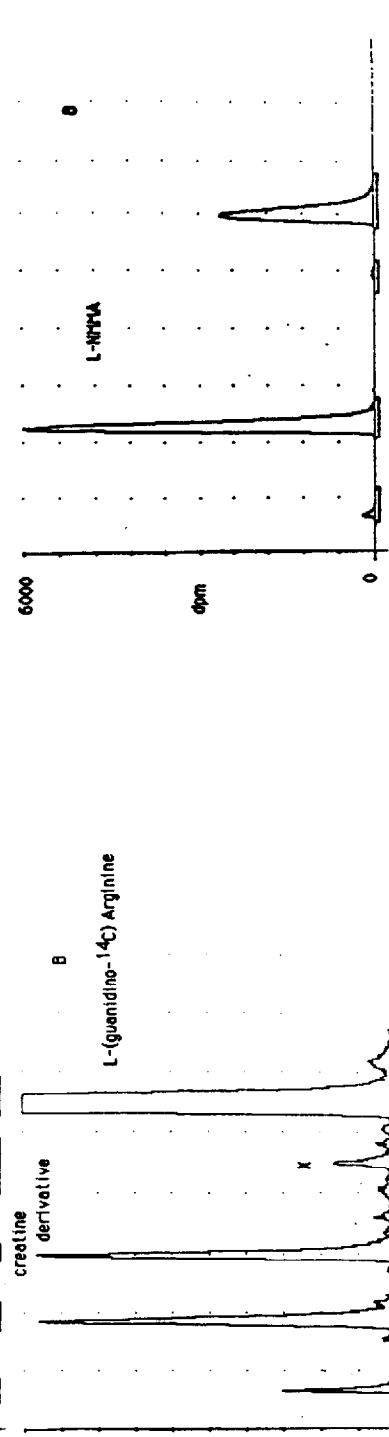

Fig. 6. Radiolabelled L-[ureido-^{14}C]citrulline-derived metabolites generated in induced EMT6 cell culture media. Added radiolabelled citrulline: 37 kBq. (A) in the absence of L-NMMA; (B) in the presence of 2.2 m*M* L-NMMA.

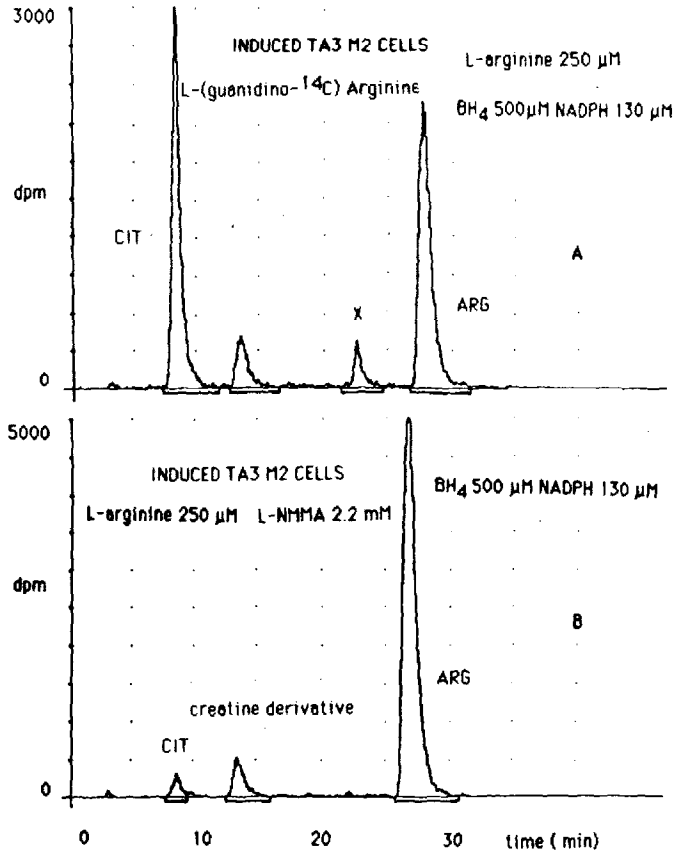

Fig. 7. HPLC analysis of cytosolic extracts from induced TA3 M2 cells. (A) In the absence of L-NMMA; (B) in the presence of 2.2 m*M* L-NMMA. Cytosolic extracts from induced cells were processed as indicated under Experimental in the presence of cofactors of the NO˙-generating pathway (NADPH, BH_4) and of 8 kBq of L-[guanidino-^{14}C]arginine.

first transformed into a less basic compound. It was very labile at alkaline pH, leading to urea as a detectable product when L-[guanidino-^{14}C]arginine was used.

DISCUSSION

We have found in culture media or cytosols of cells, in which the NO˙-generating pathway has been induced, a metabolite, X, generated at the same time as citrulline (inhibition by L-NMMA, triggering by BH_4). Metabolite X, unlike citrulline, was not found in cytotoxic macrophages, probably because of the intense metabolism of these cells (*e.g.*, very active arginase) and the lability of X. An additional product from L-arginine metabolism was found and is possibly linked to the creatine pathway. More information on this could be obtained by incubating the cells with [^{3}H]glycine (a precursor).

Metabolite X is eluted between ornithine and arginine and is therefore more

basic than ornithine and less so than arginine. It may be assumed that N^G-hydroxyarginine or N^G-oxoarginine would be less basic than arginine because of the loss of resonance energy which stabilizes the guanidinium cation. Thus, taking into account the chromatographic properties of X and the location of its radiolabelling (guanidino-^{14}C), it may be hypothesized that X is probably either N^G-hydroxyarginine or N^G-oxoarginine, the intermediates which have been postulated (Fig. 1). We are currently trying to obtain large amounts of the compound to check this hypothesis by mass spectrometry.

The metabolites of the NO$^\bullet$-generating pathway (NO$^\bullet$, N^G-hydroxyarginine) might be direct inhibitors of a key enzyme of DNA synthesis ribonucleotide reductase. This would explain the antiproliferative effects obtained through the induction of the NO$^\bullet$-generating pathway [6].

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