Analytical Biochemistry 424 (2012) 156–161

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Radiochemical high-performance liquid chromatography detection of arginine metabolism in human endothelial cells

Amelia Barilli [a], Rossana Visigalli [a], Bianca Maria Rotoli [a], Ovidio Bussolati [a], Gian Carlo Gazzola [a], Alessandro Parolari [b], Valeria Dall'Asta [a,*]

[a] Dipartimento di Medicina Sperimentale, Sezione di Patologia Generale e Clinica, Università degli Studi di Parma, 43125 Parma, Italy
[b] Dipartimento di Cardiochirurgia, Università degli Studi di Milano, and Centro Cardiologico Fondazione Monzino, IRCCS, 20122 Milano, Italy

ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form 8 February 2012
Accepted 21 February 2012
Available online 28 February 2012

Keywords:
Arginine
Nitric oxide
Arginase
Rapamycin
TNFα
Phorbol esters

ABSTRACT

Arginine is a semi-essential amino acid that plays an important role in the regulation of metabolic processes associated with several pathological/physiological conditions. In the vasculature, it mainly exerts its biological functions as a substrate of two alternative pathways: the conversion to nitric oxide (NO) by nitric oxide synthase (NOS) and the breakdown to urea and ornithine by arginase. To determine arginine metabolism, in the current study we propose an original radiochemical technique that allows the simultaneous monitoring of NOS and arginase activation within intact cells. Taking advantage of this method, we show here the consequences of different experimental conditions known to modulate endothelial homeostasis on arginine metabolism.

Arginine is a semi-essential amino acid that plays an important role in the regulation of metabolic processes associated with several pathological/physiological conditions [1]. Its availability appears to be of unique relevance in the vasculature, where it mainly exerts its biological functions as a substrate of two alternative pathways: the conversion to nitric oxide (NO)[1] and citrulline by nitric oxide synthase (NOS) and the breakdown to urea and ornithine by arginase (Fig. 1).

NO plays a crucial role in preserving vascular homeostasis; it is the most important vasodilator in vivo and inhibits functions associated with endothelial activation such as the expression of adhesion molecules, leukocyte adhesion, and platelet aggregation [2]. Conversely, a vascular increase in arginase activity under conditions of endothelial dysfunction associated with hypertension, ischemia–reperfusion, intimal hyperplasia, and aging has been described [3]; several key mediators of atherothrombosis, such as thrombin, inflammatory cytokines, and oxidized low-density lipoprotein, have also been related to the up-regulation of arginase activity in endothelial cells [4]. The maintenance of NOS/arginase balance appears to be fundamental for the correct preservation of endothelial function, and several reciprocal regulatory interactions are known to act within the cells [3].

To determine arginine metabolism, in the current study we propose an original radiochemical technique that allows the simultaneous monitoring of NOS and arginase activation within intact cells. Using this method, we show here the effects of different experimental conditions known to modulate endothelial homeostasis on arginine metabolism.

Materials and methods

Materials

Endotoxin-free fetal bovine serum (FBS), Medium 199 (M199), and RPMI-1640 medium were purchased from Celbio (Milano, Italy). L-[2,3,4-^{3}H]Arginine (45–70 Ci/mmol), L-[U-^{14}C]arginine (360 mCi/mmol), and [1,4-^{14}C]putrescine dihydrochloride (107 mCi/mmol) were obtained from PerkinElmer (Monza, Italy). Tumor necrosis factor alpha (TNFα, Alexis) was obtained from Vinci–Biochem (Firenze, Italy), whereas Sigma (Milano, Italy) was the source of phorbol 12,13-dibutyrate (PDBu), lipopolysaccharide (LPS) (*Escherichia coli,* serotype 055:B55), and all other chemicals.

* Corresponding author. Fax: +39 0521 033742.
E-mail address: valeria.dallasta@unipr.it (V. Dall'Asta).

[1] *Abbreviations used:* NO, nitric oxide; NOS, nitric oxide synthase; FBS, fetal bovine serum; M199, Medium 199; TNFα, tumor necrosis factor alpha; PDBu, phorbol 12,13-dibutyrate; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cell; EBSS, Earle's balanced salt solution; HPLC, high-performance liquid chromatography; T_r, retention time; NOHA, *N*-ω-hydroxy-L-arginine; RT–qPCR, reverse transcription–quantitative polymerase chain reaction; PKC, protein kinase C.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.02.023

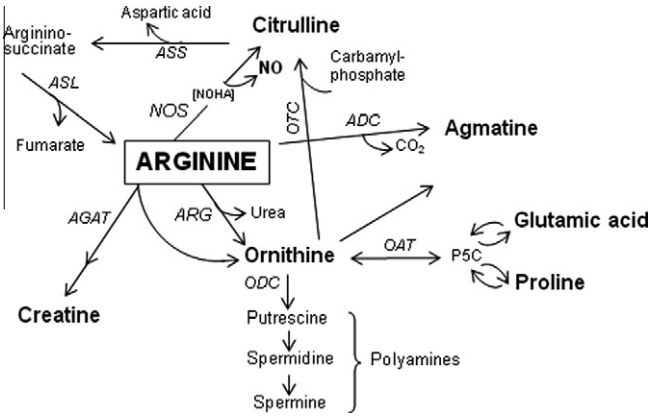

Fig.1. Arginine metabolic pathways. The cartoon highlights the enzymes involved in the main arginine metabolic pathways within endothelial cells. ADC, arginine decarboxylase; AGAT, arginine–glycine amidinotransferase; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamilase; P5C, Δ^1-pyrroline-5-carboxylate.

Cell cultures and experimental treatments

Human umbilical vein endothelial cells (HUVECs), obtained as described previously [5], were routinely grown in collagen-coated, 10-cm-diameter dishes. Cells were cultured in M199 supplemented with 20% FBS, endothelial cell growth supplement (ECGS, 37.5 μg/ml), and heparin (75 U/ml); glutamine was raised to 2 mM. The murine macrophage RAW264.7 cell line was grown in RPMI-1640 medium supplemented with 10% FBS.

Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Rapamycin and PDBu were employed at the final concentration of 100 nM in complete growth medium, whereas LPS was used at 1 μg/ml and TNFα at 10 ng/ml.

Arginine transport assay

The experiments were carried out on cells seeded onto 2-cm^2 wells of disposable 24-well trays and performed with a 30-s incubation of the cells in Earle's balanced salt solution (EBSS) containing L-[^{3}H]arginine (100 μM, 4 μCi/ml) as described previously [6]. Initial velocity of L-Arginine influx (v, L-Arg) is expressed as nmol/mg protein/min.

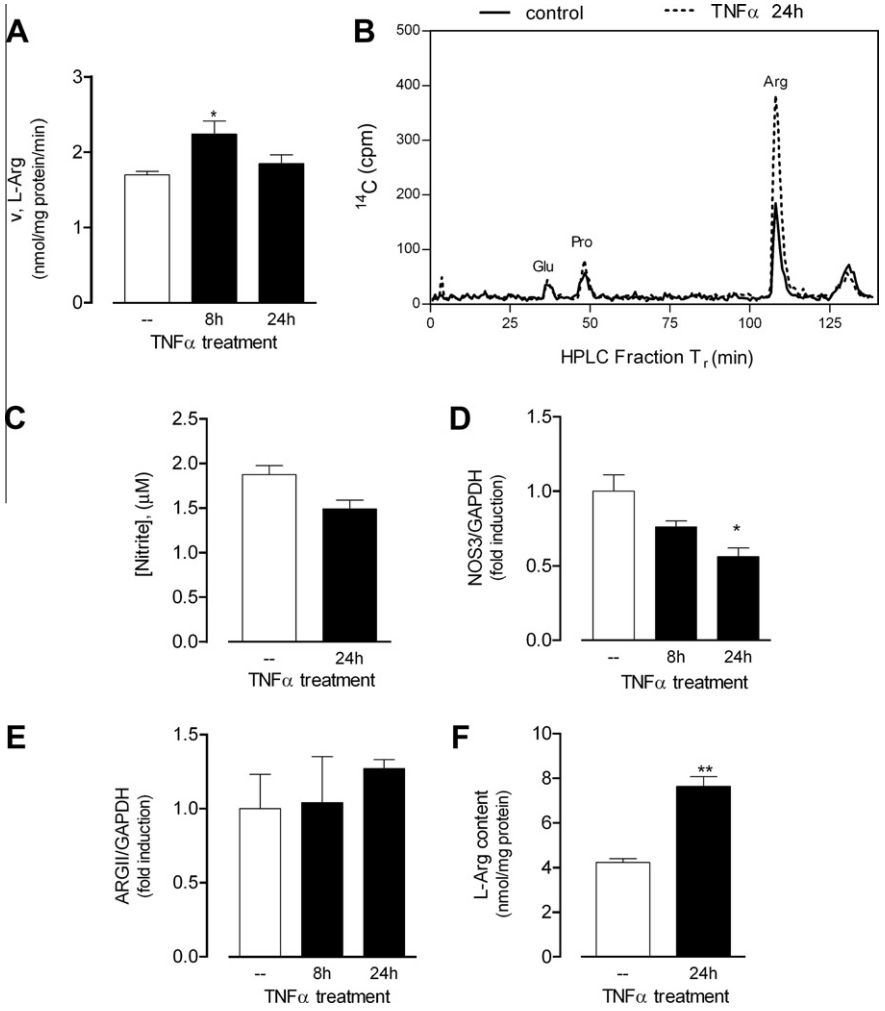

Fig.2. Effects of TNFα on arginine metabolism in HUVECs. Cells were either untreated (control) or stimulated for the indicated times with TNFα (10 ng/ml) in complete growth medium. (A) After 8 and 24 h, total L-arginine influx was assayed with a 30-s incubation of the cells in EBSS containing L-[^{3}H]arginine (100 μM, 4 μCi/ml). (B) For the determination of arginine intracellular metabolism, cells were incubated in the presence of L-[U-^{14}C]arginine (10 μCi/ml). After 24 h, an extracted amino acid pool, separated by HPLC, was collected in 192 fractions. The radioactivity in each fraction was determined and plotted as a function of retention time as described in Materials and methods. (C) The production of NO was assessed through the quantitation of nitrite in the incubation medium. (D and E) The expression of NOS3 (D) and ARGII (E) was determined through RT–qPCR analysis and is shown, after normalization to GAPDH, as fold induction relative to control untreated cells (=1). (F) L-Arginine intracellular content was measured through HPLC analysis after ninhydrin derivatization (see Materials and methods). $^*P < 0.05$ and $^{**}P < 0.01$ versus control.