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Accessibility of endothelial and inducible nitric oxide synthase to the intracellular citrulline–arginine regeneration pathway

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Abstract

This study investigates our hypothesis that argininosuccinate synthase (AS), the rate-limiting enzyme for arginine (L-arg) regeneration from citrulline (L-cit), plays a pivotal role in supplying L-arg to endothelial (eNOS), but not inducible (iNOS) nitric oxide synthase, for nitric oxide (NO) production. Transgenic rat blood–brain barrier (TR-BBB) endothelial cells were used as a model to elucidate the accessibility of the L-arg compartments for NOS isozymes. NO production via eNOS or iNOS, with or without α-methyl-DL-aspartic acid (MDLA), an AS inhibitor, was measured by a fluorometric method. NO production via eNOS was activated by the calcium ionophore A23187, while via iNOS was induced by cytokines. AS activity was assayed by the amount of argininosuccinate regenerated from radioactive aspartic acid from cell extracts. Upon increased AS activity (5.9-fold) in cells grown in L-arg-free/L-cit-supplemented medium, A23187-activated NO production also significantly increased, however cytokine-induced NO production was not detected. A23187-activated NO production was observed not only in L-arg containing medium, but also L-arg-free and L-arg-free/L-cit-supplemented medium, and was abolished by MDLA regardless of medium type. Cytokine-induced NO production was only observed in L-arg containing medium, not in L-arg-free or L-arg-free/L-cit-supplemented medium, and it was not inhibited by MDLA in the L-arg containing medium. Our results indicate that extracellular L-arg was the only L-arg pool for cytokine-induced NO production and intracellular L-arg regenerated from L-cit via AS pathway was the major L-arg pool for A23187-activated NO production in TR-BBB endothelial cells. Therefore, modulation of AS activity could be a promising strategy to selectively alter NO production via eNOS, but not iNOS.

Keywords: Argininosuccinate synthase; Nitric oxide synthases; Endothelial; Arginine; Nitric oxide; MDLA

1. Introduction

The importance of the nitric oxide synthases (NOSs) and nitric oxide (NO) is evident in physiological and pathophysiological processes. It has been suggested and much has been written about disturbances in regulation regarding inducible nitric oxide synthase (iNOS) as the likely 'damaging' producer of NO and endothelial nitric oxide synthase (eNOS) as 'protective' [1,2]. Nitric oxide, produced by eNOS, is a key mediator for maintaining the function and integrity of endothelium, such as the regulation of vascular tone, the prevention of leukocyte filtration and thrombus formation, and angiogenesis, etc. [2]. However, down-regulation of eNOS activity is associated with a number of diseases, such as atherosclerosis [3], hypertension [4] and diabetes [5]. Up-regulation of eNOS activity is correlated with tumor growth and angiogenesis in vitro and in vivo [6]. Therefore, the manipulation of eNOS activity might be a promising therapeutic strategy for a variety of diseases.

eNOS activity can be regulated by factors such as protein expression, post-translational modification, concentration of substrate and cofactor, and cellular localization [7]. The unique modification of eNOS, as compared to

Abbreviations: CAT, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; HPLC, high performance liquid chromatography; NO, nitric oxide; ADI, arginine deiminase; TR-BBB, transgenic rat-blood–brain barrier; AS, argininosuccinate synthase; L-arg, L-arginine; L-cit, L-citrulline; MDLA, α-methyl-DL-aspartic acid

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the other NOS isoforms, by myristoylation/palmitoylation of residues near the N terminus cause the particular cellular localization of eNOS in the caveolae of plasmalemma [8]. It has been shown that the mutant of the myristoyl or palmitoyl moiety of eNOS reduced NO generation, even though purified wild-type and mutant eNOS from cell extracts had identical catalytic activity [9,10]. Therefore, the association of eNOS and caveolae may render eNOS in close proximity to its substrate and cofactor for its proper functioning. Although arginine (L-arg) is the sole substrate for the NOSs, we reported previously that in vitro NO production via eNOS in transgenic rat blood–brain barrier (TR-BBB) endothelial cells is independent of extracellular L-arg, whereas iNOS solely depends on extracellular L-arg for its substrate [11]. This finding suggests that the intracellular source of L-arg, including the regenerated L-arg from argininosuccinate synthase (AS) and argininosuccinate lyase (AL), can be the substrate for eNOS, but not iNOS, in TR-BBB endothelial cells under extracellular L-arg deprivation. AS, the rate-limiting enzyme for the endogenous regenerated L-arg has been found to be co-localized with eNOS in caveolae in endothelial cells [12]. Therefore, we hypothesize that the regulation of AS activity can manipulate the NO production via eNOS.

Even though AS is recognized as a ubiquitous enzyme in mammalian tissue, the regulation of AS activity varies due to factors such as hormones, nutrients and cytokines [13]. AS has been reported to be involved in urea production in hepatocytes [14], L-arg production in enterocytes and kidney cells [15,16], NO production via eNOS in bovine aortic endothelial cells [17] and NO production via iNOS in macrophages and vascular smooth muscle cells [18,19]. However, this is the first report to elucidate the relationship between AS and NO production via eNOS and iNOS simultaneously in endothelial cells. It is crucial to understand the differential regulation of AS on NO production via eNOS and iNOS in endothelial cells. TR-BBB cells, cultured blood–brain barrier endothelial cells derived from a transgenic rat, are immortalized endothelial cells [20] and exhibit specific endothelial markers, e.g. spindle fiber-shaped morphology, von Willebrand factor and acetylated low-density lipoprotein uptake [20–22]. TR-BBB cells express eNOS, iNOS, and AS [11], and are therefore a useful model to investigate the role of AS in NO production. Here we present data to examine our hypothesis that AS, the rate-limiting enzyme for L-arg regeneration from L-cit, plays a pivotal role in the supplying L-arg to eNOS, but not iNOS, for NO production.

2. Materials and methods

2.1. Materials

Rat recombinant IFN-γ and TNF-α, nitrite standard, and Griess Reagent were purchased from Calbiochem. [^{14}C]Aspartic acid (200 mCi/mmol) was obtained from Moravek Biochemicals. Dowex 1-X8-200-400 resin was from Supelco (Bellefonte, PA). Micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). Calcium ionophore A23187, 2,3-diaminonaphthalene (DAN), AS inhibitor α-methyl-DL-aspartic acid (MDLA), as well as all other chemicals and reagents were products from Sigma Chemical Company.

2.2. Cell culture

TR-BBB endothelial cells were cultured as described previously [11]. Briefly, the cells were maintained in Dulbecco's minimal essential medium/F12 from Cellgro and supplemented with 15 μg/mL endothelial cell growth factor (ECGF), 10% heat-inactivated fetal bovine serum (FBS) and 2.5 mM L-glutamine and antibiotics, penicillin/streptomycin. The cells were cultured in culture-ware coated with type I collagen and grown in a humidified incubator at 33 °C [20]. All cell culture reagents and L-arg/L-cit-free MEMα + medium were products of GIBCO-BRL, except ECGF, which was a product of Roche Diagnostics Corporation. For experiments in this report, the L-arg-free/L-cit-free MEMα + medium is designated as 'L-arg-free medium'. L-arg-free medium supplemented with 1 mM L-arg is designated as 'control medium' and when supplemented with 1 mM L-cit/1 mM ammonium chloride, designation is 'L-arg-free/L-cit-supplemented medium'. The FBS was thoroughly dialyzed using a dialysis tubing with a molecular weight cut-off of 2 kDa, therefore the concentration of L-arg or L-cit in the serum was negligible.

2.3. AS activity assay

Cells were cultured in control medium and L-arg-free/L-cit-supplemented medium, respectively in T75 flasks for 4 days. For cytokine-treatment, cells in control medium were treated with 50 U/mL IFN-γ and 5 ng/mL TNF-α on day 3 for 24 h. AS activity in cell homogenates was determined as described in our previous paper [23] and AS enzymatic activity expressed as pmol of argininosuccinate formed from L-citr and [^{14}C]aspartic acid per min per mg protein.

2.4. Nitrite production assay

2.4.1. Effect of AS induction on NO production

To investigate the effect of up-regulation of AS activity on NO production via eNOS and iNOS, TR-BBB cells were grown under L-arg-starvation condition as a method to induce AS activity [11,24]. Successful induction of AS activity occurred under the L-arg starvation condition; therefore this experimental model was used to investigate the effect of up-regulation of AS activity on eNOS and iNOS activity.