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Recombinant arginine deiminase as a differential modulator of inducible (iNOS) and endothelial (eNOS) nitric oxide synthetase activity in cultured endothelial cells

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Abstract

Modulation of the extracellular level of arginine, substrate for nitric oxide synthetases, is a promising modality to alleviate certain pathological conditions where excess nitric oxide (NO) is produced. However, complications arise, as only preferential inhibition of the inducible nitric oxide synthetase (iNOS), but not endothelial nitric oxide synthetase (eNOS), is desired for the treatment of NO over-production. We investigated the effect of arginine deprivation mediated by a recombinant arginine deiminase (rADI) on the activity of iNOS and eNOS in an endothelial cell line, TR-BBB. Our results demonstrated that cytokine-induced NO production depends on the extracellular arginine as substrate. However, if sufficient citrulline is present in the medium, A23187-activated NO production by eNOS does not rely on extracellular arginine. Treatment with rADI can markedly inhibit cytokine-induced NO production *via* iNOS, but not A23187-activated NO production *via* eNOS. Our results also showed that the decrease of NO production by iNOS could be achieved by depleting arginine from the medium even under the conditions that would up-regulate iNOS expression. Thus, rADI appears to be a novel selective modulator of iNOS activity that may be a used as a tool in the study of pathological disorders where NO over-production plays a key role.

Keywords: Recombinant arginine deiminase; iNOS inhibitor; eNOS; Argininosuccinate synthetase

1. Introduction

Several inflammatory conditions, such as intestinal inflammation [1,2], rheumatoid arthritis [3], sepsis [4,5], and diabetes [6], are related to an over-expression of iNOS in macrophages and endothelial cells. Therefore, the suppression of iNOS activity is a rational approach for the treatment or prevention of many diseases [6,7]. However, the therapeutic use of iNOS inhibitors is complicated by the presence of several NO producing isozymes. In particular, NO, produced by the eNOS is an important mediator for many cellular processes in endothelial cells, as well as for other neighboring cell types, such as smooth muscle cells [8]. Thus, adverse responses have been observed in studies where a high bolus dose of the non-specific nitric oxide synthetases inhibitor, nitro-L-arginine-methyl ester (L-NAME), was administered for the treatment of sepsis; possibly due to the inhibition of eNOS activity [9,10]. Similarly, iNOS activity has been associated with injuries in liver and lung during septic shock, and evidence indicates that eNOS can prevent the damage in both organs [4,11]. Conceivably, drugs that can selectively inhibit iNOS, with little or no effect on eNOS, would be highly desirable for the treatment of iNOS-related diseases [12].

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Abbreviations: iNOS, inducible nitric oxide synthetase; eNOS, endothelial nitric oxide synthetase; NO, nitric oxide; rADI, recombinant arginine deiminase; TR-BBB, transgenic rat-blood–brain barrier; AS, argininosuccinate synthetase; L-arg, L-arginine; L-cit, L-citrulline.

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Even though great efforts have been made by the pharmaceutical industry to develop iNOS-specific inhibitors, no such drug is currently available.

Arginine is the substrate for NO synthetases that produce NO. Arginine deiminase (ADI) has been shown to reduce NO production in macrophages *in vitro* [13,14] and *in vivo* [15]. However, these observations in macrophages were determined after stimulation by cytokines or lipopolysaccharides, thus generating NO *via* the induced isoform, iNOS. A measurement of the contribution of NO production by the constituent isoform, eNOS, has not been determined. Thus far, there is no report on the differential effect of ADI on either iNOS and eNOS activity or on the underlying mechanisms for the ADI-mediated iNOS inhibition. Since eNOS plays an important role in regulating many vascular and physiological processes, an elucidation of the effects of ADI on iNOS and eNOS can be very useful in the development of NOS-specific inhibitors.

In this report, we investigated the differential effects of rADI on iNOS and eNOS activities utilizing cultured blood–brain barrier endothelial cells derived from a transgenic rat, TR-BBB, as a paradigm. The TR-BBB cell line is an immortalized endothelial cell line that has been shown to exhibit many of the biomarkers characteristic of normal endothelial cells [16,17]. We further demonstrated that TR-BBB cells are capable of expressing both iNOS and eNOS and, therefore, are a useful model to elucidate the effects of rADI on these two isozymes in endothelial cells.

2. Materials and methods

2.1. Materials

The rADI was purified to homogeneity and enzyme activity determined in our laboratory [18]. Rat recombinant IFN-γ and TNF-α, nitrite standard, and Griess Reagent were purchased from Calbiochem. [^{14}C]Arginine (264 mCi/mM) and [^{14}C]aspartic acid (200 mCi/mM) were obtained from Moravek Biochemicals. TLC plates (silica gel 60 F_{254}) were purchased from EM Science. Dowex 1-X8-200-400 resin was from Supelco. Micro BCA protein assay reagent kit was obtained from Pierce. The secondary horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody was from Bio-Rad. Molecular mass markers were purchased from Amersham Biosciences. Calcium ionophore A23187, 2,3-diaminonaphthalene (DAN), mouse monoclonal anti-eNOS, and mouse monoclonal anti-iNOS antibodies, as well as all other chemicals and reagents were products from Sigma Chemical Company.

2.2. Cell culture

TR-BBB endothelial cells were isolated from the blood–brain barrier of temperature-sensitive simian virus 40 large T-antigen transgenic rats and were cultured as described previously [17,19]. Briefly, TR-BBB endothelial cells were maintained in Dulbecco's minimal essential medium/F12 (DMEM/F12) from Cellgro and supplemented with 15 μg/mL endothelial cell growth factor (ECGF) and 10% heat-inactivated fetal bovine serum (FBS), 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° which is the permissive temperature for the cell proliferation [16,17]. All cell culture reagents and arginine/citrulline-free MEM alpha (+) medium were products of GIBCO-BRL, except ECGF, which was a product of Roche Diagnostics Corporation. For experiments in this report, the arginine/citrulline-free MEM alpha (+) medium supplemented with 1 mM L-arginine (L-arg) is designated as 'control medium' and when supplemented with 1 mM L-citrulline (L-cit)/1 mM ammonium chloride designation is 'arginine-free medium'.

2.3. Nitrite production assay

2.3.1. The effect of rADI on nitrite production via iNOS

Cells were seeded (2×10^4 cells/well) into 24-well culture plates in 10% FBS DMEM/F12 + ECGF. After 4 hr, 50 U/mL IFN-γ, 5 ng/mL TNF-α, and various rADI concentrations from 0 to 1.0 mU/mL were added to the cultures. After a 4-day incubation, nitrite concentration in the supernatant was measured by the Griess method [20]. Briefly, in a 96-well plate, 50 μL of Griess Reagent R1 and of Griess Reagent R2 were added to 100 μL of supernatant from each sample well. After 10 min, the absorbance at 570 nm was read using a Dynatech MR 700 plate reader. The cells were rinsed with cold PBS and the protein content in each well was determined by Pierce Protein Kit and results presented as nmol of nitrite in the cell culture supernatant per mg protein.

2.4. The effect of arginine deprivation on nitrite production via iNOS

Cells were seeded (2×10^4 cells/well) into 24-well culture plates in control medium or arginine-free medium. All media contained 10% dialyzed (>2 kDa) FBS. At 4-hr post-seeding, cytokines and rADI were added to the cultures, then after a 4-day incubation, nitrite concentration and protein content was determined as previously described [21].

2.5. The effect of rADI or arginine deprivation on nitrite production via eNOS

Cells were seeded (1×10^5 cells/well) into 6-well culture plates in control medium or arginine-free medium. On day 3, cells were treated with 1 mU/mL rADI. Separately, fresh medium was also treated (1 mU/mL rADI) and incubated. After 24 hr (day 4), the cells were rinsed twice

with PBS and media replaced with the pre-incubated medium. After 30-min incubation, 10 μM calcium ionophore, A23187, was added and the cells incubated for an additional 30 min. The nitrite concentration in the supernatant was measured by a modified fluorometric method [22,23]. After centrifugation, 100 μL of freshly prepared DAN (0.05 mg DAN/mL in 0.62 M HCl) was added to 900 μL of cell culture supernatant and mixed immediately. After 15-min incubation at room temperature, the reaction was terminated with 50 μL of 2.8 M NaOH and then each sample diluted with 10 mM NaOH to bring the final volume up to 2 mL/vial. The samples were measured using the excitation wavelength of 375 nm and emission wavelength of 415 nm with a Hitachi F-2000 Fluorescence Spectrophotometer. Nitrite concentrations were calculated from a nitrite standard curve and adjusted by protein content in each well (determined by the Pierce Protein Kit) and presented as nmol of nitrite in the cell culture supernatant per mg protein.

2.6. Analyses of L-arg and L-cit in cell culture medium

Cells were seeded (2×10^4 cells/well) into 24-well culture plates in control medium. After 4 hr, various rADI concentrations at 0, 0.01, 0.1, and 1.0 mU/mL were added to the cultures. Eight microliters of medium from days 0, 1, 2, and 4 was spotted onto TLC plates and developed in chloroform/methanol/ammonium hydroxide/water, 0.5:4.5:2.0:1.0 (v/v/v/v) as described previously [24]. Radioactivity of each cm^2 per lane was determined by scintillation counter and results expressed as the percent control.

2.7. Argininosuccinate synthetase (AS) activity assay

AS activity in cell homogenates was determined as described in our previous paper [21] and AS enzymatic activity expressed as pmol of argininosuccinate formed from citrulline and [^{14}C]aspartic acid/min/mg protein. Briefly, cell monolayers treated with 1.0 mU/mL rADI were rinsed with PBS, homogenized, and 200 μL of the cell supernatant assayed for AS activity where the amount of [^{14}C]argininosuccinate formed was quantified using anion exchange chromatography.

2.8. Western blot assay

TR-BBB cells were seeded (1×10^5/well) into 6-well culture plates. For NOS blotting, cells were treated with cytokines and rADI on day 1, as described above in the method section for NO production *via* iNOS. Proteins were extracted and separated by 7.5% SDS–PAGE gel and blotted onto nitrocellulose membranes as described [25] using antibodies against iNOS (1:3000), eNOS (1:2000), and actin (1:10,000). Horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (1:10,000) was used as secondary antibody, and peroxidase activity was detected by enhanced chemiluminescence (ECL, Amersham).

2.9. Statistical analysis

All values are mean ± SD. The significant difference was evaluated by ANOVA followed by the modified *t*-test according to Bonferroni, when appropriate. Values of $P < 0.05$ were accepted as significant.

3. Results

3.1. Effect of rADI on NO production via iNOS

Experiments were performed to determine the range of rADI concentrations that would inhibit NO production *via* iNOS in TR-BBB endothelial cells. Results showed that the *in vitro* NO production induced by cytokines (iNOS) was abrogated by rADI treatment (Fig. 1). An approximate 6-fold increase of NO production in TR-BBB endothelial cells was observed with a combined treatment of IFN-γ (50 U/mL) and TNF-α (5 ng/mL), 398.0 ± 10.4 nmol/mg vs. 61.4 ± 13.4 nmol/mg ($P < 0.001$), with and without cytokine treatment, respectively. Concentrations between 0.01 and 1.0 mU/mL of rADI strongly inhibited the iNOS-mediated NO production in a dose–response manner decreasing from 398.0 ± 10.4 nmol/mg to 88.4 ± 20.0 nmol/mg ($P < 0.001$), respectively. The highest concentration of rADI reduced the NO to a level comparable to that of control (60 nmol/mg). Additionally, after the 4-day treatment, rADI was removed by replacing with fresh medium containing arginine and incubating for an additional 4 days with or without cytokines, then NO production measured. TR-BBB cells were able to recover NO production *via* iNOS through cytokine stimulation (223.3 ± 11.4 nmol/mg).

To assess the effect of arginine deprivation on NO production by iNOS, TR-BBB cells were incubated for 4 days in arginine-free medium supplemented with 1 mM L-cit/1 mM ammonium chloride. The inhibition of NO production by arginine deprivation or in combination with cytokines and rADI treatment is shown in Fig. 2. Upon cytokine treatment, nitrite concentration in control medium (with arginine) was measured at 64.0 ± 17.8 nmol/mg, whereas in arginine-free medium there was no detectable nitrite. Induction of NO production by cytokines and its inhibition by rADI (1.0 mU/mL) occurred only in control medium containing arginine and not in arginine-free, citrulline-supplemented medium.

3.2. Effect of rADI on NO production by eNOS

The residual NO production observed in the cytokine-induced rADI-treated cells, as well as the low level in the control cells, was further investigated to determine whether

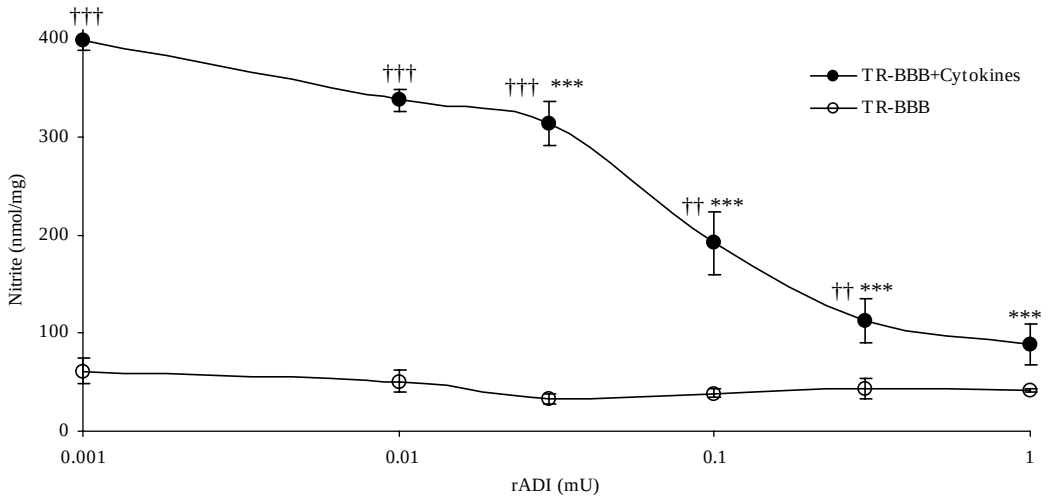

Fig. 1. The effect of rADI in TR-BBB cells on NO production *via* iNOS. Cells were seeded in 24-well collagen-coated culture plates and treated with rADI at the indicated concentrations and incubated for 4 days at 33°, 95% air/5% CO_2. Cytokines, 50 U/mL IFN-γ, and 5 ng/mL TNF-α were added on day 1. Nitrite concentration was determined using a commercially available Griess Assay kit. Error bars represent standard deviations (N = 3). $^{***}P < 0.001$ within the cytokine-treated group, each value compared to 0.001 mU/mL rADI (control). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ cytokine-treated vs. no cytokines for each concentration of rADI.

the activity was due to iNOS or eNOS. A23187 increases the permeability of biological membranes to Ca^{2+} in intact cells; therefore, due to the Ca^{2+}/calmodulin dependence of eNOS activity, this constitutive enzyme is activated by A23187 whereas iNOS is not. As shown in Fig. 3, rADI, at a dose of 1.0 mU/mL, had no inhibitory effect on the A23187-activated NO production. Compared to the control, A23187 treatment enhanced the nitrite production from 0.93 ± 0.13 nmol/mg to 3.2 ± 0.6 nmol/mg ($P < 0.05$). This 3.5-fold increase of NO production, however, was not affected by the addition of rADI (nitrite concentration, 5.1 ± 0.3 nmol/mg), indicating that eNOS is not sensitive to rADI inhibition. In arginine-free medium, A23187-activated nitrite production was significantly higher than that in arginine-containing medium, i.e. 5.9 ± 1.4 nmol/mg vs. 3.2 ± 1.0 nmol/mg, respectively ($P < 0.05$).

3.3. Effect of rADI on L-arg in the cell culture medium

To determine the amount of L-arg and L-cit in the cell culture medium treated with rADI, TLC was performed. Using the described developing solvent system, R_f values of L-arg and L-cit are 0.35 and 0.82, respectively, which is consistent to those values previously reported [24]. On day 0, control was considered as 100% L-arg and 0% L-cit when corrected for background (Fig. 4). At 1.0 mU/mL, rADI dramatically decreased the amount of L-arg present to only 5% of the original concentration in 1 day with a corresponding increase of L-cit in the medium. At 0.1 mU/mL, the effect of rADI was more gradual; where the amount of L-arg present was 63, 19, and 4% on days 1, 2, and 4, respectively. The increase in L-cit formed was again complimentary to the observed decrease of L-arg. At the lowest

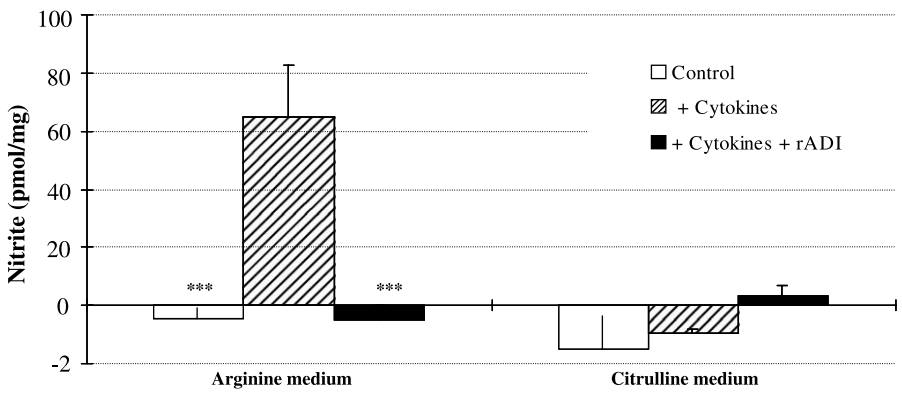

Fig. 2. The effect of arginine deprivation in TR-BBB cells on NO production *via* iNOS. Cells were seeded into 24-well collagen-coated culture plates in complete or arginine-free (supplemented with 1 mM L-cit/ammonium chloride) medium and treated with cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α) and 1.0 mU/mL rADI, incubated at 33°, 95% air/5% CO_2. On day 4, nitrite concentration was measured using a commercially available Griess Assay kit. Error bars represent standard deviations (N = 3). $^{***}P < 0.001$ cytokine-treated vs. control or cytokines + rADI.

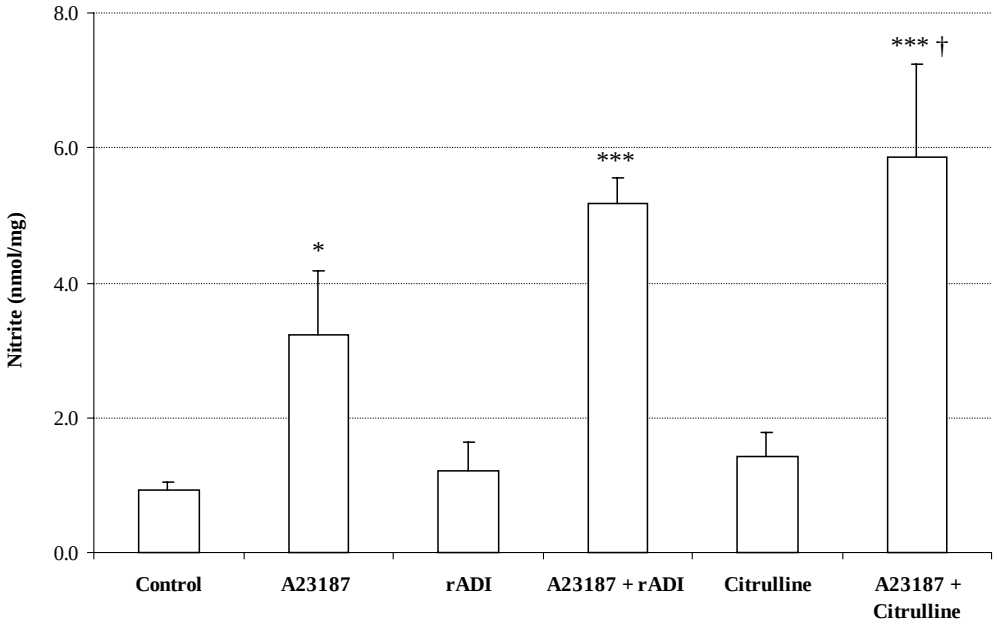

Fig. 3. The effect of rADI on NO production *via* eNOS in TR-BBB cells. Cells were seeded into 6-well collagen-coated culture plates, incubated at 33°, 95% air/5% CO_2, and then treated with 1.0 mU/mL rADI on day 3. On day 4, calcium ionophore A23187 was added. NO concentration was determined by measuring nitrite concentration in the culture medium by the fluorometric assay described in Section 2. Error bars represent standard deviations (N = 3). $^{*}P < 0.05$, $^{***}P < 0.001$ arginine control group vs. each respective treatment. $^{\dagger}P < 0.05$ A23187 + arginine vs. A23187 + citrulline.

rADI concentration of 0.01 mU/mL tested, on day 4 only a moderate 25% decrease in L-arg was observed and the amount of L-cit increased 16%.

3.4. The effect of rADI on AS activity

To investigate the effect of rADI on AS activity, cells were treated with 1.0 mU/mL of rADI for 24 hr prior to the determination. The data in Fig. 5 demonstrate that AS activity increased from 29.1 to 195.3 pmol/min/mg (6.7-fold) with cytokine treatment (50 U/mL IFN-γ and 5 ng/mL TNF-α). Treatment with 1.0 mU/mL rADI alone increased AS activity from 29.1 to 209.2 pmol/min/mg (7.2-fold), while treatment with arginine-free medium supplemented with citrulline increased from 29.1 to 173.0 pmol/min/mg (5.9-fold).

3.5. Effect of rADI on cytokine-induced iNOS and eNOS protein expression

To determine whether or not the expression of NOS isozyme proteins could be modulated by rADI in TR-BBB cells, Western blot analysis was performed for iNOS and eNOS. Quantification of the bands was determined and normalized with that of β-actin. In this representative blot, TR-BBB cells expressed a minimal level of iNOS protein when incubated in the medium without the addition of cytokines (Fig. 6A, lane 1, control). Upon the addition of

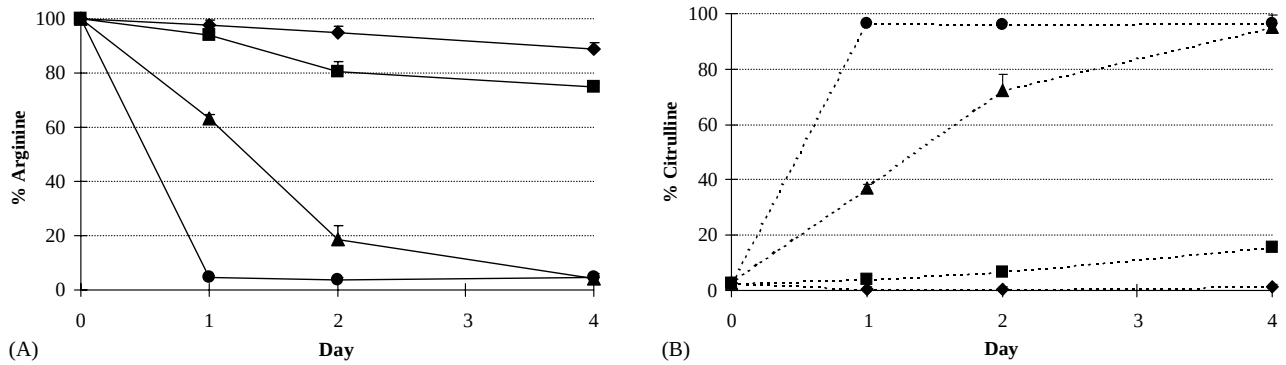

Fig. 4. Analyses of the amount of (A) L-arg and (B) L-cit in cell culture medium treated with rADI. Cells were seeded (2×10^4 cells/well) into 24-well culture plates in control medium (containing radiolabeled L-arg) and then treated with rADI. TLC analysis of culture medium on the indicated day was performed to determine the amount of L-arg and L-cit and results expressed as the percent the original amount of L-arg present in the culture medium.

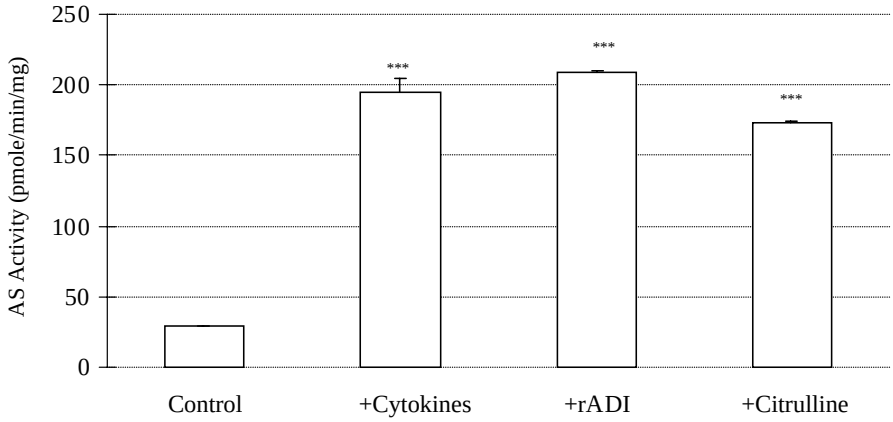

Fig. 5. The effect of cytokines, rADI, and arginine-free citrulline-supplemented medium on AS activity. Cells were seeded into T75 collagen-coated culture flasks and incubated at 33°, 95% air/5% CO_2. Cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α) and 1.0 mU/mL rADI were added on day 3. AS activity was assayed as described in Section 2 and the results expressed as the mean ± SD (N = 3). $^{***}P < 0.001$ control vs. each respective treatment.

either rADI or cytokines (Fig. 6A, lanes 2 and 3), the level of iNOS protein expression increased compared to control (1.5 ± 0.9-fold and 4.0 ± 1.3-fold increase, respectively). However, upon concomitant treatment of cytokines and rADI (Fig. 6A, lane 4), a 6.1 ± 2.2-fold increase above control was observed in iNOS protein expression ($P < 0.01$). On the other hand, with rADI or cytokine treatment, the amount of eNOS protein is 2.2 ± 0.9-fold and 1.2 ± 0.1-fold (Fig. 6B, lanes 2 and 3) greater than control (Fig. 6B, lane 1) and a 2.8 ± 1.1-fold increase was observed with concomitant treatment (Fig. 6B, lane 4). There was no statistically significant difference in the amount of eNOS protein expression between treatments.

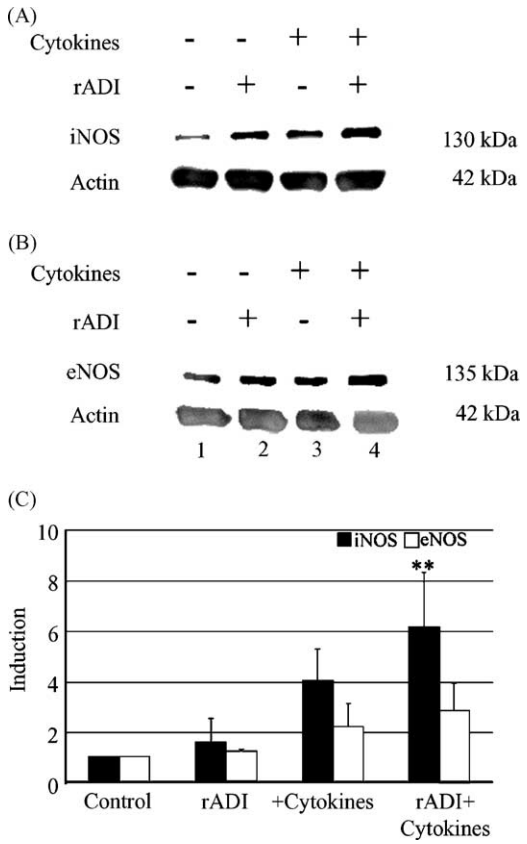

Fig. 6. The effects of rADI on expression of iNOS and eNOS in TR-BBB cells. Cells were treated with cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α,) and 1.0 mU/mL rADI on day 1. Protein extracts were separated on 7.5% SDS–PAGE gels and transferred to nitrocellulose membranes, then probed with iNOS or eNOS antibody. (A) and (B) Photographs of a representative Western blot of three independent experiments. (C) Quantitative data of desitometric analysis of the stained bands, mean ± SD (N = 3), $^{**}P < 0.01$.

4. Discussion

Our results indicate that cytokine-induced NO production in TR-BBB cells, i.e. the iNOS activity, was abolished by rADI in a dose-dependent manner (Fig. 1). Correspondingly, the activity of rADI decreases the amount of arginine present in the culture medium with concurrent increase in the amount of citrulline (Fig. 4). The suppression of cytokine-induced NO production by rADI is consistent with recent findings by others in macrophages *in vitro* [14] and mice *in vivo* [15]. We also found that cytokine-induced NO production in TR-BBB cells occurred only in complete arginine-containing medium, but not in arginine-free/citrulline-containing medium. Furthermore, if arginine is replenished to the culture medium of rADI-treated TR-BBB cells, a recovery of NO production *via* iNOS can be achieved. Therefore, the mechanism of rADI-mediated inhibition of cytokine-induced NO production in TR-BBB cells is most likely due to the extracellular conversion of arginine into citrulline by rADI. In this regard, it is interesting that A23187-activated NO production, or the calcium-dependent eNOS activity, in TR-BBB cells is not decreased either by rADI treatment or arginine-free, citrulline-containing medium (Fig. 3). Based on these findings, it becomes apparent that NO production *via* iNOS depends exclusively on the extracellular arginine as substrate. Whereas, NO production *via* eNOS does not rely on

extracellular arginine when sufficient citrulline is present in the medium.

Another possibility for the decrease in NO production may be due to down-regulation of iNOS by rADI treatment. However, this possibility is ruled out by the Western blot analyses revealing an up-regulation, rather than a down-regulation, of iNOS protein levels in rADI-treated TR-BBB cells (Fig. 6). The apparent lack of effect by rADI on the eNOS protein steady-state level may also indicate that the selective NO inhibition is mediated *via* rADI arginine depletion, rather than repression of iNOS biosynthesis. However, further studies are needed, as the literature regarding the effect of cytokines on eNOS protein expression is limited and contradictory due to heterogeneity of endothelial cells from various vascular beds [26]. Nevertheless, taken together, these results suggest that the decrease of NO production by rADI is due to the deprivation of extracellular arginine, the substrate of iNOS.

In contrast to iNOS, either extracellular arginine or citrulline can be used as a substrate for NO production *via* A23187-activated eNOS. Of particular interest is our observation that citrulline is as effective as, and possibly even better than, arginine as a substrate for NO production by eNOS. As shown in Fig. 3, the amounts of NO production *via* A23187-activated eNOS are similar in medium containing either 1 mM L-arg + rADI or 1 mM L-cit. This difference in utilization of citrulline by eNOS and iNOS to produce NO further supports the assumption of the compartmentalization of the intracellular arginine pools [27]. Additionally, it has been reported that eNOS and arginine regeneration enzymes, i.e. AS and argininosuccinate lyase (AL), are co-localized in plasmalemmal caveolae [28]. Thus, the co-localization of these enzymes may provide a unique compartment for eNOS to utilize regenerated arginine to produce NO. However, the caveolar compartment is inaccessible to iNOS and, therefore, iNOS is unable to utilize the arginine regenerated from citrulline in order to produce NO. In the citrulline–arginine regeneration pathway, AS is the rate-limiting enzyme [29]. A correlation has also been reported between the resistance to the antiproliferative activity of rADI and AS in various cell lines [21]. Indeed, our results showed that a 7-fold increase of AS activity was observed in rADI-treated TR-BBB cells (Fig. 5). Such a discernible increase in AS activity may also explain why NO production by eNOS in rADI-treated TR-BBB cells is slightly higher than in the controls (Fig. 5).

Overall, our results demonstrate that the conversion of extracellular arginine to citrulline by rADI can markedly attenuate NO production by iNOS, because the activity of this enzyme is largely dependent upon the extracellular arginine concentration. On the other hand, the presence of citrulline, instead of arginine, in rADI-treated medium allows a normal activity of eNOS to be maintained due to the co-localization, in caveolae, of eNOS with the key arginine-regenerating enzyme, AS. It is noteworthy that endogenously generated arginine in hepatocytes has been shown to be directly channeled from AS to AL and, subsequently, from AL to arginase in the urea cycle [30]. Conceivably, such a channeling event may also occur between AS, AL, and eNOS in endothelial cells, and may explain how eNOS can selectively utilize the citrulline produced by rADI to generate NO. Therefore, rADI appears to be a selective inhibitor of NO production *via* iNOS, but not eNOS. In addition, our results also demonstrated that rADI treatment can up-regulate the expression of several arginine-related proteins, including iNOS and AS. Therefore, ADI appears to act paradoxically on the regulation of iNOS activity, i.e. it increases the gene expression, yet decreases the NO production, of this enzyme. This increase in gene expression requires further investigation for possible development of rADI for use in the study of selective inhibition of increased iNOS-mediated NO production manifested in many diseases.

Acknowledgments

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References

[1] Oudkerk Pool M, Bouma G, Visser JJ, Kolkman JJ, Tran DD, Meuwissen SG, Pena AS. Serum nitrate levels in ulcerative colitis and Crohn's disease. Scand J Gastroenterol 1995;30:784–8.

[2] Levine JJ, Pettei MJ, Valderrama E, Gold DM, Kessler BH, Trachtman H. Nitric oxide and inflammatory bowel disease: evidence for local intestinal production in children with active colonic disease. J Pediatr Gastroenterol Nutr 1998;26:34–8.

[3] Borderie D, Hilliquin P, Hernvann A, Kahan A, Menkes CJ, Ekindjian OG. Nitric oxide synthase is expressed in the lymphomononuclear cells of synovial fluid in patients with rheumatoid arthritis. J Rheumatol 1999;26:2083–8.

[4] Lee RP, Wang D, Kao SJ, Chen HI. The lung is the major site that produces nitric oxide to induce acute pulmonary oedema in endotoxin shock. Clin Exp Pharmacol Physiol 2001;28:315–20.

[5] Piepot HA, Groeneveld AB, van Lambalgen AA, Sipkema P. The role of inducible nitric oxide synthase in lipopolysaccharide-mediated hyporeactivity to vasoconstrictors differs among isolated rat arteries. Clin Sci (Lond) 2002;102:297–305.

[6] Du Y, Smith MA, Miller CM, Kern TS. Diabetes-induced nitrative stress in the retina, and correction by aminoguanidine. J Neurochem 2002;80:771–9.

[7] Preiser JC, Zhang H, Vray B, Hrabak A, Vincent JL. Time course of inducible nitric oxide synthase activity following endotoxin administration in dogs. Nitric Oxide 2001;5:208–11.

[8] Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. J Pathol 2003;199:8–17.

[9] Fischer LG, Horstman DJ, Hahnenkamp K, Kechner NE, Rich GF. Selective iNOS inhibition attenuates acetylcholine- and bradykinin-induced vasoconstriction in lipopolysaccharide-exposed rat lungs. Anesthesiology 1999;91:1724–32.

[10] Schwartz D, Brasowski E, Raskin Y, Schwartz IF, Wolman Y, Blum M, Blantz RC, Iaina A. The outcome of non-selective vs. selective nitric oxide synthase inhibition in lipopolysaccharide treated rats. J Nephrol 2001;14:110–4.
[11] Morikawa A, Kato Y, Sugiyama T, Koide N, Chakravortty D, Yoshida T, Yokochi T. Role of nitric oxide in lipopolysaccharide-induced hepatic injury in D-galactosamine-sensitized mice as an experimental endotoxic shock model. Infect Immun 1999;67:1018–24.
[12] Strunk V, Hahnenkamp K, Schneuing M, Fischer LG, Rich GF. Selective iNOS inhibition prevents hypotension in septic rats while preserving endothelium-dependent vasodilation. Anesth Analg 2001; 92:681–7.
[13] Dillon B, Holtsberg F, Ensor M, Bomalaski J, Clark M. Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. Med Sci Monit 2002;8:BR248–53.
[14] Noh EJ, Kang SW, Shin YJ, Kim DC, Park IS, Kim MY, Chun BG, Min BH. Characterization of mycoplasma arginine deiminase expressed in *E. coli* and its inhibitory regulation of nitric oxide synthesis. Mol Cell 2002;13:137–43.
[15] Thomas JB, Holtsberg FW, Ensor CM, Bomalaski JS, Clark MA. Enzymic degradation of plasma arginine using arginine deiminase inhibits nitric oxide production and protects mice from the lethal effects of tumour necrosis factor alpha and endotoxin. Biochem J 2002;363:581–7.
[16] Hosoya K, Tetsuka K, Nagase K, Tomi M, Saeki S, Ohtsuki S, Takanaga H, Yanai N, Obinata M, Kikuchi A, Okano T, Terasaki T. Conditionally immortalized brain capillary endothelial cell lines established from a transgenic mouse harboring temperature-sensitive simian virus 40 large T-antigen gene. AAPS Pharm Sci 2000;2:E27.
[17] Terasaki T, Hosoya K. Conditionally immortalized cell lines as a new in vitro model for the study of barrier functions. Biol Pharm Bull 2001;24:111–8.
[18] Beloussow K, Wang L, Wu J, Ann D, Shen WC. Recombinant arginine deiminase as a potential anti-angiogenic agent. Cancer Lett 2002;183: 155–62.
[19] Hosoya KI, Takashima T, Tetsuka K, Nagura T, Ohtsuki S, Takanaga H, Ueda M, Yanai N, Obinata M, Terasaki T. mRNA expression and transport characterization of conditionally immortalized rat brain capillary endothelial cell lines; a new in vitro BBB model for drug targeting. J Drug Target 2000;8:357–70.
[20] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[21] Shen LJ, Lin WC, Beloussow K, Shen WC. Resistance to the antiproliferative activity of recombinant arginine deiminase in cell culture correlates with the endogenous enzyme, argininosuccinate synthetase. Cancer Lett 2003;191:165–70.
[22] Miles AM, Wink DA, Cook JC, Grisham MB. Determination of nitric oxide using fluorescence spectroscopy. Methods Enzymol 1996;268: 105–20.
[23] Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11–6.
[24] Hilderman RH, Casey TE, Pojoga LH. P(1),P(4)-Diadenosine 5′-tetraphosphate modulates l-arginine and l-citrulline uptake by bovine aortic endothelial cells. Arch Biochem Biophys 2000;375:124–30.
[25] de Frutos T, de Miguel LS, Garcia-Duran M, Gonzalez-Fernandez F, Rodriguez-Feo JA, Monton M, Guerra J, Farre J, Casado S, Lopez-Farre A. NO from smooth muscle cells decreases NOS expression in endothelial cells: role of TNF-alpha. Am J Physiol 1999;277:H1317–25.
[26] Zhang J, Patel JM, Li YD, Block ER. Proinflammatory cytokines downregulate gene expression and activity of constitutive nitric oxide synthase in porcine pulmonary artery endothelial cells. Res Commun Mol Pathol Pharmacol 1997;96:71–87.
[27] Closs EI, Graf P. Cationic amino acid transporters (CATs). Targets for the manipulation of NO-synthase activity? Pharm Biotechnol 1999; 12:229–49.
[28] Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC. Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. Nitric Oxide 2001;5:187–97.
[29] Hattori Y, Campbell EB, Gross SS. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. J Biol Chem 1994;269:9405–8.
[30] Cheung CW, Cohen NS, Raijman L. Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. J Biol Chem 1989; 264:4038–44.