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In silico analysis of arginine catabolism as a source of nitric oxide or polyamines in endothelial cells

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Summary. We use a modeling and simulation approach to carry out an in silico analysis of the metabolic pathways involving arginine as a precursor of nitric oxide or polyamines in aorta endothelial cells. Our model predicts conditions of physiological steady state, as well as the response of the system to changes in the control parameter, external arginine concentration. Metabolic flux control analysis allowed us to predict the values of flux control coefficients for all the transporters and enzymes included in the model. This analysis fulfills the flux control coefficient summation theorem and shows that both the low affinity transporter and arginase share the control of the fluxes through these metabolic pathways.

Keywords: Arginine – Nitric oxide – Ornithine – Polyamines – Metabolic control analysis

Introduction

L-Arginine is a conditionally essential amino acid for adult mammals, since it should be supplied by diet under physiological or pathological conditions in which its requirement exceeds its velocity of production. Its metabolic relevance is stressed by the fact of being a precursor for a wide variety of biomolecules playing very important metabolic, regulatory, and physiological roles. Figure 1 summarizes the main metabolic pathways in which arginine is involved. Exogenous arginine is taken up via several isoforms of the y^+ system for cationic amino acids (Grillo and Colombatto, 2004a). Arginine is the immediate precursor of urea as a substrate of arginase in the urea cycle. It also participates in the synthesis of creatine, which in phosphorylated state plays a relevant role in muscle bioenergetics. Arginase pathway bifurcates at the level of ornithine, either leading to L-proline biosynthesis (required for collagen synthesis) or to the polyamine synthetic pathway. Arginine/ornithine-derived polyamines are ubiquitous aliphatic polycations with pleiotropic biological effects, including a relevant regulatory role in macromolecular synthesis and cell proliferation rate (Medina et al., 2003, 2005). Direct decarboxylation of arginine could produce agmatine, which can act as a biosignaling molecule and as an intermediate in an alternative pathway of polyamine synthesis (Grillo and Colombatto, 2004b; Morris, 2004). In the last fifteen years, arginine has attracted renewed attention, since it is the immediate precursor of nitric oxide (NO) through the nitric oxide synthase reaction. NO is a bio-active gas involved in many processes, including neurotransmission, immune response, vasodilation and adhesion of platelets and leukocytes (Wu and Morris, 1998; Grillo and Colombatto, 2004a; Bronte and Zanovello, 2005). Therefore, arginine metabolism is particularly relevant in physio-pathological contexts such as the regulation of immune responses (Bronte and Zanovello, 2005) and endothelial/vascular health/disease (Cooke and Tsao, 1997; Gornik and Creager, 2004).

In spite of its relevance, arginine catabolism is not fully understood. A comprehensive understanding of arginine metabolism biological functions should require new holistic approaches, as claimed by the emergent systems biology (Kitano, 2002; Medina et al., 2005). Metabolic flux control analysis is frequently used to access global regulatory features of whole metabolic pathways (Fell, 1997). On the other hand, mathematical modeling and simulation could provide an operational way to explore in silico these

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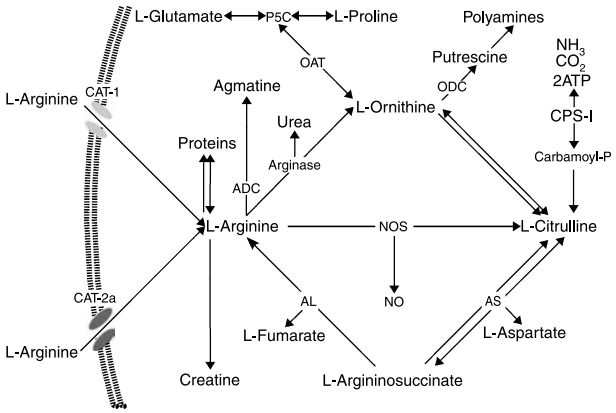

Fig. 1. Overview of arginine catabolism. *ADC* Arginine decarboxylase; *AL* argininesuccinate lyase; *AS* argininesuccinate synthase; *carbamoyl-P* carbamoyl-phosphate; *CAT-1* high affinity arginine transporter; *CAT-2a* low affinity arginine transporter; *CPS-I* carbamoyl-phosphate synthetase I; *NO* nitric oxide; *NOS* nitric oxide synthase; *OAT* ornithine aminetransferase; *ODC* ornithine decarboxylase; *P5C* pyrroline-5-carboxylate

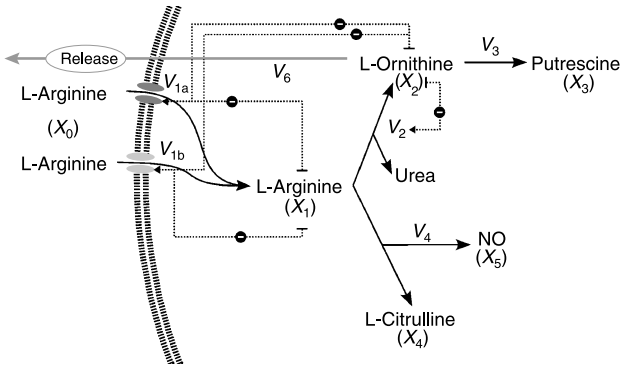

Fig. 2. Simplified model of the catabolic branch of arginine as a precursor of NO and polyamines, including described inhibitions and release. See text for details

global regulatory features, as shown by relevant mathematical models of several well-known metabolic pathways published recently (Nijhout et al., 2004; Reed et al., 2004; Yang et al., 2005). In this work, we describe the modeling and in silico analysis of the branch of arginine metabolism leading to either NO or polyamines in aorta endothelial cells (see Fig. 2), based on known enzymology and available, experimentally measured intermediate metabolite concentrations and kinetic data on the components included in the model. The model allows for the use of metabolic flux control analysis to determine the relative contribution of the different components to the regulation of the flux through the system.

Materials and methods

Description of the model

We delimited our system of study as the portion of arginine metabolism leading from external arginine to either NO or putrescine (the first argi-

Table 1. Rate equations for enzymes and transports included in the model

Equations and parameters	Description
Arginine transport $\nu_{tranp} = \frac{[Arg_{ex}]}{K_m^{Hat} + [Arg_{ex}]} \cdot \frac{V_{\max}^{Hat}}{1 + \left(\frac{[Orn]}{K_{iO}^{Hat}}\right) + \left(\frac{[Arg_{in}]}{K_m^{Hat}}\right)} + \frac{[Arg_{ex}]}{K_m^{Lat} + [Arg_{ex}]} \cdot \frac{V_{\max}^{Lat}}{1 + \left(\frac{[Orn]}{K_{iO}^{Hat}}\right) + \left(\frac{[Arg_{in}]}{K_m^{Lat}}\right)}$	Equation (with parameters taken from Brenda enzyme database[a]) that assembles the activities of the high and low activity transporters
Arginase $\nu_{Arg} = \frac{V_{\max}^{Arg} \cdot [Arg_{in}]}{K_m^{Arg} \cdot \left(1 + \left(\frac{[Orn]}{K_{iO}^{Arg}}\right)\right) + [Arg_{in}]}$	Michaelis-Menten equation for competitive inhibition by ornithine
ODC $\nu_{ODC} = \frac{V_{\max}^{ODC} \cdot [Orn]}{K_M^{ODC} + [Orn]}$	Michaelis-Menten equation
NOS $\nu_{NOS1} = \frac{V_{\max}^{NOS1} \cdot [Arg_{in}]}{K_m^{NOS1} + [Arg_{in}]}$	Michaelis-Menten equation
Ornithine efflux $\nu_{effluxO} = \frac{V_{\max}^{effl.Hat}}{1 + \left(\frac{[Arg_{ex}]}{K_m^{Hat}}\right)} \cdot \frac{[Orn]}{K_{iOrn}^{Hat} \cdot \left(1 + \left(\frac{[Arg_{in}]}{K_m^{Hat}}\right)\right) + [Orn]} + \frac{V_{\max}^{effl.Lat}}{1 + \left(\frac{[Arg_{ex}]}{K_m^{Lat}}\right)} \cdot \frac{[Orn]}{K_m^{effl.Lat} \cdot \left(1 + \left(\frac{[Arg_{in}]}{K_m^{Lat}}\right)\right) + [Orn]}$	Equation (with parameters taken from Brenda enzyme database[a]) that assembles the activities of the high and low activity transporters

[a] Source: www.brenda.uni-koeln.de

nine/ornithine-derived polyamine). Due to the relevance of arginine metabolism to endothelial function and to the availability of a relatively homogenous set of experimental data obtained using aorta endothelial cells, we adopted our model to the known enzymology of arginine metabolism in endothelial cells. Two transporters of the y^+ family are considered to model arginine uptake and ornithine efflux: the high affinity CAT-1 and the low affinity CAT-2a transport systems (Mann et al., 2003; Grillo and Colombatto, 2004a; Verrey et al., 2004; Rotoli et al., 2005). Three enzymes are included in the model: arginase, which splits arginine to urea plus ornithine; ornithine decarboxylase, which decarboxylates ornithine to putrescine; and nitric oxide synthase, wihich splits arginine to citrullin plus NO. Although endothelial cells express two arginase isozymes, mitochondrial arginase II is not included in the model, since aorta endothelial cells only express it as a response to lipopolysaccharide activation (Buga et al., 1996). Basic described inhibitions are also taken into account in the model, including inhibition of both transporters by both internal arginine and ornithine, and inhibition of arginase by ornithine. For all the transporters and enzymes included in the model, a very simple Michaelian kinetic behavior is considered.

Since aorta endothelial cells from different sources are easily cultured and components of culture media can be controlled, we used external arginine concentration as the control parameter of the system. Table 1 shows the rate equations for enzymes and transporters included in the model. Arginine uptake and ornithine efflux velocities are considered to be the addition of velocities through both the high and low affinity transporters. Table 2 shows the differential equations for the two time-dependent variables (internal arginine and ornithine) considered in the model. Table 3 shows the range of experimentally-determined values described in the literature for the different kinetic parameters of the model and the values selected to be included in the model. The values of the parameters describing ornithine efflux are considered to be equal to those describing arginine uptake. The selected external concentration of arginine is that contained in DMEM culture medium, frequently used to culture endothelial cells (Baydoun et al., 1990).

Table 2. Differential equations for time-dependent variables included in the model

Time dependent variables	Differential equation
Arg_{in} Inner Arginine	$\frac{d[Arg_{in}]}{dt} = \nu_{transp} - \nu_{Arg} - \nu_{NOS}$
Orn Ornithine	$\frac{d[Orn]}{dt} = \nu_{Arg} - \nu_{ODC} - \nu_{effluxO}$

The models were implemented in Perl language (http://www.perl.com/). Simulations were carried out by the iterarative Euler method, with a time increment of 0.01 min per iteration. In all the simulations, steady state was considered to be reached when the change of any variable was lower than 10^{-10} within an interval of 3 h (18000 iterations). To simulate the response of the system to changes in the control parameter, different simulations were carried out changing the values of external arginine concentration within a four order of magnitude range (from 10 μM to 10 mM), as experimentally carried out (Arnal et al., 1995).

Consideration of metabolic flux control analysis coefficients and theorem

Metabolic flux control analysis theory is an extension of classical enzymology to a metabolic context, allowing for a deterministic and quantitative description of the contribution of all metabolites and modulators (both called as ''structural elements'') and enzymes or functional proteins (called ''operator elements'') taking part in the control of a metabolic pathway (Fell, 1997). This theory is built on the ground of a number of

Table 3. Model parameter values and initial values

Description	Symbol	Model	Range	References
High affinity transport	$V_{\max}^{Hat}$	$160.5\,\mu M \cdot min^{-1}$	$36.8–161.4\,\mu M \cdot min^{-1}$	Kikuta et al. (1998)
	K_m^{Hat}	70 μM	68–140 μM	Kikuta et al. (1998)
	$K_{i,Orn}^{Hat}$	380 μM	$360 \pm 35\,\mu M$	Bogle et al. (1996)
Low affinity transport	$V_{\max}^{Lat}$	$420\,\mu M \cdot min^{-1}$	$112–435\,\mu M \cdot min^{-1}$	Kikuta et al. (1998)
	K_m^{Lat}	847 μM	847 μM	Kikuta et al. (1998)
Arginase	$V_{\max}^{Arg}$	$110\,\mu M \cdot min^{-1}$	$40–116\,\mu M \cdot min^{-1}$	Buga et al. (1996)
	K_m^{Arg}	1500 μM	1000–3000 μM	Buga et al. (1996)
	$K_{i,Orn}^{Arg}$	1000 μM	1000 μM	Daghigh et al. (1994), Buga et al. (1996)
ODC	V_{max}^{ODC}	0.013 μM	0.12×10^{-3}–$0.013\,\mu M \cdot min^{-1}$	Osterman et al. (1995)
	K_M^{ODC}	60 μM	60 μM	Osterman et al. (1995)
Ornithine efflux	$V_{\max}^{effl.Hat}$	$160.5\,\mu M \cdot min^{-1}$	–	
	$V_{\max}^{effl.Lat}$	$420\,\mu M \cdot min^{-1}$	–	
	$K_m^{effl.Hat}$	380 μM	–	
	$K_m^{effl.Lat}$	847 μM	–	
NOS	$V_{\max}^{NOS}$	$1.33\,\mu M \cdot min^{-1}$	$1.2–16\,\mu M \cdot min^{-1}$	Hecker et al. (1994), Gerber et al. (1997)
	K_m^{NOS}	16 μM	3.9–22 μM	Buga et al. (1996), Gerber et al. (1997), Leber et al. (1999)
Metabolite parameter	$[Arg_{ex}]$	330 μM[a]	50–100 μM	Marquez et al. (1989), Darblade et al. (2001), Cynober (2002)

[a] Concentration in DMEM culture medium

axioms and considering several coefficients (related to local and systemic properties), yielding several theorems (deduced and demonstrated on the basis of the axioms). In this study, we have taken into account two systemic coefficients and a theorem.

Flux control coefficient is described as the percentual variation of the flux through a pathway induced by a percentual variation in one of the operator elements of the system:

$C^{J}_{Ei} = (\delta J/J)/(\delta Ei/Ei)$, with J is the flux and Ei is an operator element of the system. This coefficient gives information on the relative contribution of every enzyme or transporter of a pathway to the control of the flux through it.

Response coefficient is described as the percentual variation of the flux through a pathway induced by a percentual variation in one of the structural elements of the system:

$R^{J}_{x} = (\delta J/J)/(\delta Ei/Ei)$, with J is the flux and x is a structural element of the system. This coeffcient shows the relative importance of any modulator or metabolite in a pathway.

The summation theorem of flux control coefficients establishes that in any metabolic pathway under steady state conditions the total sum of the C^{J}_{Ei} for all the Ei of the system has to be equal to 1.

To determine the values of flux control coefficients, percentual changes of the activities of each enzyme or transporter were introduced in the model and simulations yielded new data allowing for the estimation of the correspondent percentual changes in fluxes. In the case of response coefficients, we only determined the response of the system to percentual changes in the value of the control parameter, external arginine concentration.

Results

Simulations of our model from initial conditions yielded a physiological steady-state condition in less than 1 h, as shown both in the evolution of inner arginine and ornithine concentrations (Fig. 3A) and enzyme activities (Fig. 3B). These values are within the range of experimental values so far reported (Table 4). This steady state was considered as the basal condition for the application of flux control analysis theory (see below). Furthermore, additional simulations changing the value of the control parameter, external arginine, up to four orders of magnitude (from 10 μM to 10 mM) gave also rise to steady state conditions after no more than 100 min (Fig. 4). At least within three orders of magnitude, the steady state conditions as determined by inner arginine levels (Table 5) were within the described range of values in a previous experimental study (Arnal et al., 1995).

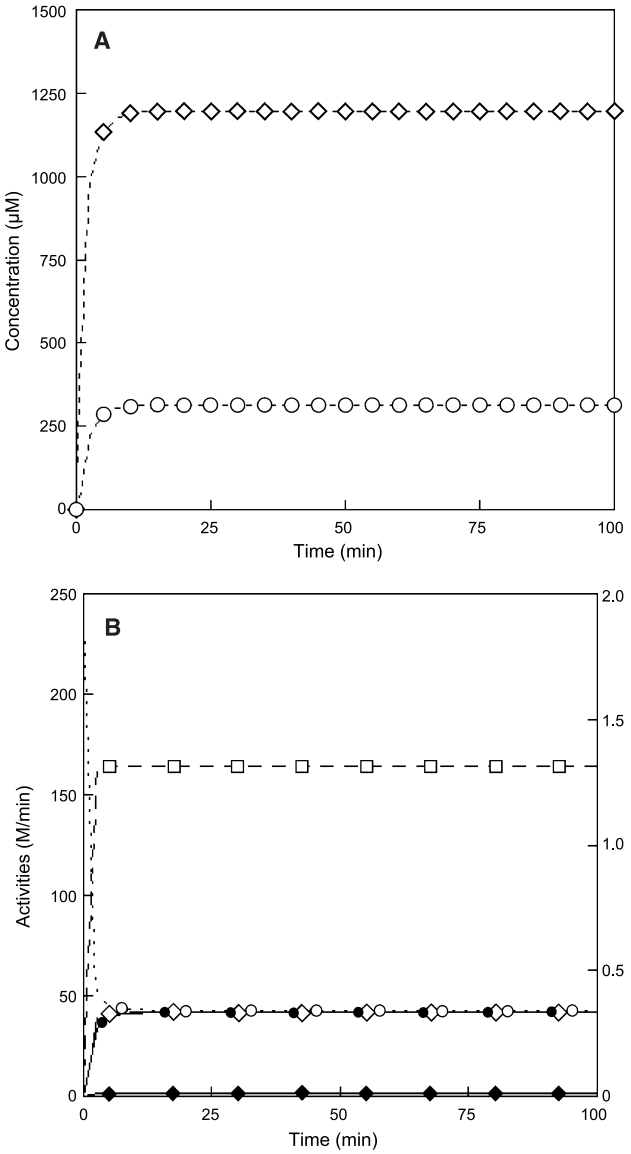

Fig. 3. Simulations of the model yield a physiological steady state. **A** Evolution of the concentrations of inner arginine (triangles) and ornithine (diamonds). **B** Evolution of activities: high affinity transporter (open triangles); low affinity transporter (open squares); arginase (diamonds); nitric oxide synthase (closed grey squares); ornithine decarboxylase (closed triangles); efflux (closed black squares)

Table 4. Simulation of the model yields a physiological basal steady-state

Description	Symbol	Model	Range	References
Inner arginine concentration	$[Arg_{in}]$	1196 (μM)	100–800 in-culture 800–2000 in-vivo (μM)	Baydoun et al. (1990), Arnal et al. (1995)
Ornithine concentration	$[Orn]$	314 (μM)	220–410 (μM)	Marquez et al. (1989), Casey et al. (2000)
Transport activity	ν_{transp}	43 (μM/min)	34.1–80.7 (μM/min)	Kikuta et al. (1998), Casey et al. (2000)
Arginase activity	ν_{Arg}	42 (μM/min)	293 (μM/min)	Buga et al. (1996)
ODC activity	ν_{ODC}	0.01 (μM/min)	0–0.17 (μM/min)	Morrison and Seidel (1995)
NOS activity	ν_{NOS}	1.31 (μM/min)	1.33 (μM/min)	Hecker et al. (1994)
Efflux activity	$\nu_{EffluxO}$	42 (μM/min)	–	–

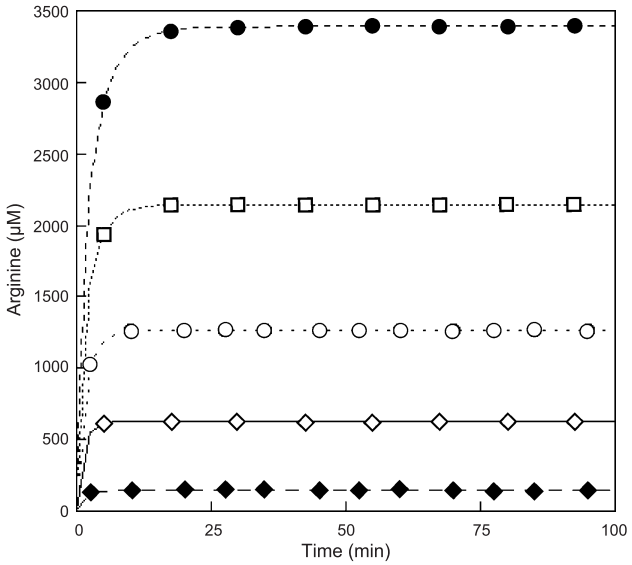

Fig. 4. The model is robust. Inner arginine steady state values monotonically increase with increasing external arginine levels (10, 100, 330, 1000 and 10000 μM)

Table 5. Simulations fit well with experimental data within 3 orders of magnitude of external arginine

Outside arginine (μM)	Inner arginine (in-vivo) (μM)[a]	Inner arginine (in-silico) (μM)
10	200–340	142
100	350–610	628
330	800–2000	1268
1000	2160–3440	2147
10000	9200–11400	3391

[a] Data from Arnal et al. (1995)

Application of flux control analysis theory to the basal conditions achieved in simulations of the model allowed us to determine flux control coefficient for all the operator elements included in the model (Table 6), namely, the high affinity transproter, the low affinity transporter, arginase, ornithine decarboxylase, nitric oxide synthase and the combined action of transporters for ornithine efflux. Furthermore, Table 6 shows that the summation theorem for flux control coefficients is fulfilled.

The study of the response of the system to changes in the control parameter (external arginine) was a second application of flux control analysis theory in this work (Fig. 5). The response curve showed a three-steps pattern, with a fast decay in the R^J_x value from 0.001 to 0.25% eternal arginine (taking 0.33 mM arginine as 100%), a wide range of external arginine values (from 25 to 250%) with small changes in the response coefficient value, and a second region of fast decay in the response coefficient value at external arginine concentrations higher than 250%.

Discussion

In spite of the paramount biological importance of arginine/ornithine derived polyamines and nitric oxide (Wu and Morris, 1998; Medina et al., 2003; Grillo and Colombatto, 2004a), the relative contribution of their biosynthetic enzymes to the regulation of arginine catabolism pathway is not well described. To get new insights in this topic by in silico application of flux control analysis was a final goal of this work. To achieve this goal, we had previously to build a model of the pathway and to show that the model yielded physiological steady states and was robust.

We built an extremely simple model of arginine catabolism, taking only into account the branches leading to NO and putrescine (Fig. 2). Nonetheless, in spite of its simplicity, our model managed to simulate properly the behavior of this pathway in endothelial cells, yielding a basal steady state with physiological values of metabolite concentrations and enzyme activities (Fig. 3 and Table 4) within the range of available experimental data. Furthermore, our model was very robust, as shown by its ability to reach steady states for a range of external arginine concentrations covering four orders of magnitude (Fig. 4). This in silico experiment simulated the experiments carried out by Arnal et al. to determine the effect of changes in extracellular arginine concentrations on intracellular arginine levels (Arnal et al., 1995). The values of inner arginine concentration once the steady states were reached in our in silico experiment were within the experimental range of values for extracellular arginine concentration values of 0.01, 0.1 and 1 mM (Table 5). Only for an external arginine concentration value as high as 10 mM (far away from the physiological situation, since plasma concentrations of arginine are in the 60–100 μM range) (Marquez et al., 1989; Darblade et al., 2001; Cynober, 2002), the steady state value of inner arginine yielded by

Table 6. Flux control coefficients for enzymes and transporters included in the model

	H.A.T	L.A.T	Arginase	ODC	NOS	Efflux	Summation
$C_E{}^J$	0.0964	0.3604	0.3548	0.0000	0.0173	0.1937	0.9992

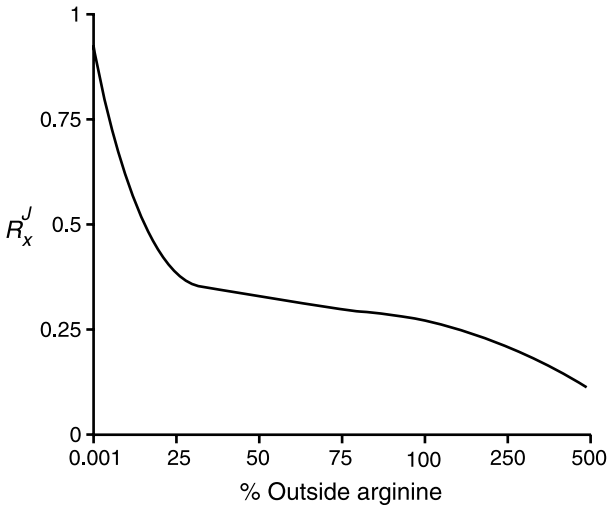

Fig. 5. Curve showing the evolution of the response coefficient with changes in the levels of external arginine (taking the 330 μM concentration included in the initial conditions as 100%)

simulations of our model was out of the range of reported experimental values (Arnal et al., 1995).

As expected from systems biology approaches, the predictions of this simple model could contribute to propose new hypothesis to be experimentally tested. This has been the case, since our model has allowed us to apply flux control analysis, obtaining results that could be experimentally tested in the future. The use of mathematical models represents a helpful tool to minimize the actual number of real experiments to only those producing interesting results, according to the predictions of the model simulations. The deduced flux control coefficient values, obtained for the operator elements included in the model (Table 6) show us that endothelial cell arginine catabolic rate is mainly controled by the low affinity arginine transporter and arginase (C^J_{Ei} values of 0.360 and 0.355, respectively), with a remarkable contribution of ornithine efflux ($C^J_{Ei} = 0.197$). It should be stressed that, although ornithine decarboxylase and nitric oxide synthase are usually taken as key enzymes in the biosynthetic pathways of polyamines and NO, respectively, their contribution to the control of the flux through arginine catabolic pathway is negligible. The fulfillment of the summatory theorem for flux control coefficients can be taken as a support for the confidence on the results obtained in the simulations of our model.

Finally, the curve describing the changes in the response coefficent with changing values of the control parameter (external arginine) shows that the system is more sensible to these changes at low external concentrations of arginine (<80 μM) and becomes almost insensitive to changes in external arginine under conditions well above (80–800 μM) the usual physiological range.

Since metabolic networks have been shown to be hierarchical and modular, relative simple extensions of a metabolic model are allowed by aggregation of additional modules. This modular approach should provide in the future more comprehensive and detailed models of arginine metabolism.

Acknowledgements

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