

DETERMINANTS OF CELLULAR L-ARGININE TRANSPORT

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg 2012.

DECLARATION

I declare that this is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. The work contained in this thesis has not been submitted for any other degree or examination in this University or any other University.

Margaretha Johanna Nel
on this 6th day of September 2012.

I certify that the studies contained in this thesis have the approval of the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa. The Ethics number is M120154.

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In memory of my loving parents

Frederik Petrus Nel

1928-1994

and

Margaretha Johanna Nel (Visser)

1929-1997

to whom I will always be grateful and who are sorely missed.

“Aan my eerbare ouers wie geleef het ten spyte van omstandighede, ten koste

van hulself, ter wille van my en my broer wie baie na aan my hart is”

PRESENTATIONS ARISING FROM THIS STUDY

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Nel MJ., Woodiwiss AJ., Van Zyl RL., Candy GP. 2008a. Arginine uptake by human endothelial cells. The 36th Annual Meeting of the Surgical Research Society of Southern Africa.

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Nel MJ., Woodiwiss AJ., Van Zyl RL., Candy GP. 2009a. Elucidation of arginine uptake by endothelial cells. The 37th Annual Meeting of the Surgical Research Society of Southern Africa.

Nel MJ., Woodiwiss AJ., Van Zyl RL., Candy GP. 2009b. The effects of arginine and homocyst(e)ine on nitric oxide production in ECV₃₀₄ and HUVEC vascular endothelial cells. The 37th Annual Meeting of the Surgical Research Society of Southern Africa.

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Nel MJ., Woodiwiss AJ., Candy GP. 2012a. Modelling of cellular arginine uptake by more than one transporter. The 17th Biennial congress of the Southern African Hypertension Society.

Nel MJ., Woodiwiss AJ., Candy GP. 2012b. Non-linear modeling of cationic amino acid uptake into HUVEC and ECV₃₀₄ cells allows distinction between transporters. The 17th Biennial congress of the Southern African Hypertension Society.

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- 2) Nel MJ., Woodiwiss AJ., Candy GP. 2012b. Non-linear modeling of cationic amino acid uptake into HUVEC and ECV₃₀₄ cells allows distinction between transporters. *Cardiovasc J SA* 2012.
- 3) Nel MJ., Woodiwiss AJ., Candy GP. 2012c. Homocystine inhibits y^+L arginine transport in human umbilical vein endothelial cells without affecting y^+ transport. *Cardiovasc J SA* 2012.

NOTE ON REFERENCING IN TEXT

In the text of this thesis, referencing indicates the first author, except in cases of the same first author publishing more than one article in one year with the same co-authors. In such cases, I have included the second and the third author for the purpose of clarity and an 'a' or 'b' after the year of publication, in the case of referring to the authors: Baylis and White & Christensen.

ABSTRACT

One of the potential causes of hypertension is endothelial dysfunction associated with a decreased production of the vasodilator nitric oxide (NO). Possible factors which may contribute to the reduced NO production include increased reactive oxygen species (eg. superoxides); increased concentrations of homocysteine; or decreased concentrations of L-arginine (cationic amino acids). L-arginine, the precursor of NO, not only increases the bioavailability of NO by increasing its production; but also by reducing the inactivation of NO by superoxides. In patients with hypertension, although fasting plasma L-arginine concentrations are elevated, L-arginine supplementation has been shown to decrease blood pressure. A possible explanation for these data may be that L-arginine uptake into cells is impaired and therefore would not be available for NO production. Indeed, studies have shown that cellular uptake of L-arginine is reduced in lymphocytes from patients with hypertension and individuals genetically predisposed to developing hypertension. However, elucidating the kinetics of L-arginine uptake into endothelial cells is fundamental to determine whether L-arginine uptake is indeed impaired.

Previous studies have shown that the uptake of cationic amino acids into endothelial cells is mediated by the high affinity/low rate y^+ L transporter and the low affinity/high rate y^+ transporter. However, data on the kinetics, the relative contribution and physiological importance of the individual transporters in cells expressing more than one transporter, are inconsistent; as most studies determining the uptake of radiolabelled amino acids have assumed Michaelis-Menten kinetics and have calculated constants from Lineweaver-Burk reciprocal plots and Eadie-Hofstee plots. Another approach was therefore required to overcome the limitations and assumptions made in these studies. My first aim was therefore to determine the kinetics of L-arginine uptake into endothelial cells using a general non-linear approach, which allows initial rates of uptake by more than one transporter to be determined and importantly includes the actual concentrations of

both the trace radiolabelled and unlabelled amino acid in the model. Furthermore, using this approach no assumptions are made regarding the type of inhibition and the concentrations of inhibitors (or activators) could be included in the model. As the model was additive, the theoretical contribution of uptake by each transporter could be modelled.

The present study used raw, rather than transformed data, in non-linear regression analysis to characterize the kinetics of L-arginine uptake into cells. I modelled the initial high affinity/low capacity and low affinity/high capacity uptake of trace L- $[^3\text{H}]$ arginine by two transporters into ECV₃₀₄ and umbilical cord vein endothelial cells in the presence of a range of unlabelled L-arginine and modifiers using GraphPad Prism. The contribution of uptake by individual transporters was modelled and showed that leucine inhibited the individual transporters differently and that the inhibition was not necessarily competitive. *N*-ethylmaleimide inhibited only y^+ transport and 2-amino-bicyclo-[2,2,1]-heptane-2-carboxylic acid may be a potential inhibitor of y^+L transport. Only the absence of sodium reduced L-arginine uptake by y^+L transport and reduced the K_m' , whereas reducing sodium decreased L-arginine uptake by y^+ transport without affecting the K_m' . This non-linear modelling approach allows more than one transporter to be modelled, overcomes many of the assumptions made in reported studies and by using raw, rather than transformed data, avoids the errors inherent in methods deriving constants from the linearization of the uptake processes following Michaelian kinetics. The results of this study therefore provide explanations for discrepancies in the literature and suggest that this modelling approach better characterises the kinetics of amino acid uptake into cells.

Having elucidated the kinetics of L-arginine uptake into endothelial cells, I was then equipped to explore possible factors which could impair L-arginine uptake in hypertension. In this regard, although increases in total plasma homocysteine were thought to play a role in hypertension; large prospective clinical trials to reduce total plasma homocysteine by vitaminB_{6/12}/folate supplementation, have

failed to show beneficial effects on vascular outcomes. The effects of homocysteine on the vasculature were attributed to the reactive free sulphydryl group; however only a fraction (1.5 – 4%) of total plasma homocysteine is actually present as the free reduced sulphydryl (-SH or thiol) form. In comparison, free oxidized homocysteine, present as the disulphide, homocystine and the mixed disulphide (with cysteine) accounts for 20 – 30% of total plasma homocysteine. In the absence of a clear mechanism by which homocysteine causes vascular disease, one of the other species making up the total homocysteine may be contributing to vascular disease through a different mechanism which may not involve the free sulphydryl group.

Earlier studies demonstrated (in isolated nephrons) that the homocysteine disulphide, homocystine, shared the same membrane transporter as L-arginine (the precursor of NO), and competed for uptake with L-arginine. These studies may suggest that increased homocystine concentrations, by inhibiting L-arginine transport, and hence reducing intracellular L-arginine concentrations, may impact on NO production in other cell types. Therefore, the second aim of my study was to determine the effects of homocystine on cellular L-arginine uptake and hence on NO production.

The uptake of labelled L-[³H]arginine was measured in confluent, L-arginine depleted HUVEC and ECV₃₀₄ cells with unlabelled L-arginine, without or with homocystine and modifiers. The kinetic constants were determined in Graphpad Prism using a described non-linear model of uptake for two transporters acting simultaneously. The NO specific fluorescent DAF-2 dye was used to detect NO production by the cells. Elevated physiological concentrations of 2.5µM homocystine significantly inhibited L-arginine uptake by 90% by y⁺L transport in both HUVEC (p<0.0005) and in ECV₃₀₄ cells (p<0.05). Homocystine reduced the *K_{ma}* of y⁺L transport in HUVEC (<0.0001) affecting uptake in a competitive-like manner. Pre-incubation of the ECV₃₀₄ cells with L-arginine was able to reverse this inhibition by homocystine. In contrast, homocystine increased uptake by y⁺

transport in HUVEC ($p < 0.01$). Under the experimental conditions used, effects of homocystine on the rate of NO production could not be shown. By demonstrating that homocystine nearly abolishes L-arginine uptake by γ^+L transport in both HUVEC and ECV₃₀₄ cells, these data provide a mechanism as to how homocystine may affect L-arginine concentrations. These data would support studies to determine the association between homocystine concentrations and cardiovascular disease.

Lastly, although angiotensin-converting enzyme inhibitors (ACEI's, as well as angiotensin II receptor antagonists) but not other classes of antihypertensive agents, have been shown to decrease oxidative stress and increase NO availability independent of blood pressure lowering effects, the mechanism is not clear. The ability of ACEI's to decrease oxidative stress and enhance NO production has been attributed in part to the sulfhydryl groups present in some, but not all, ACEI's. Hence the mechanisms of the effects of ACEI's on NO production warrant further investigation, as it is possible that L-arginine transporters may play a role by enhancing L-arginine uptake into cells, and thereby increasing NO production.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibitor
ACEI's	angiotensin converting enzyme inhibitors
ADMA	asymmetric dimethyl L-arginine
Ado	adenosine
AdoHcy	adenosylhomocysteine
ADP	adenosine diphosphate
AMT	2-amino-5,6-dihydro-6-methyl-4H-1,3thiazine
ApoA-1	Apolipoprotein A-1
Asp	aspartine
ATCC	American Tissue Cell Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BAEC	bovine aortic endothelial cells
BASMC	bovine aortic smooth muscle cells
BCH	2-amino-2-norbornane-carboxylic acid or β -2-aminobicyclo(2,2,1)heptanes-2-carboxylic acid
BH ₄	tetrahydrobiopterin
Bq	Becquerel
°C	degrees centigrade
Ca ²⁺	calcium
CAT	cationic amino acid transporter (y ⁺)
CD62E	cluster designation 62 endothelial
cDNA	copy deoxyribonucleic acid
CHF	congestive heart failure
Ci	Curi
C.I.	Confidence Interval
Cl ⁻	Chloride
cGMP	cyclic-guanosine monophosphate

cm ²	squared centimeter
cNOS	constitutive nitric oxide synthase
CO ₂	carbon dioxide
cpm	counts per minute
CβS	cystathionine-β-synthase
d	deci
Da	Dalton
DA	diacetate
DAF	diamino fluorescein
DAF-DA	diamino fluorescein diacetate
DDAH	dimethylarginine dimethylamine hydrolase
DEA/NO	diethylamine NONOate sodium salt hydrate
DMEM	Dulbecco's Minimum Essential Medium
DNA	deoxyribonucleic acid
EA.hy926	human endothelial vein cells fused with human lung cancer cells (hybrid number 926)
ECV ₃₀₄	transformed human endothelial cord vein cells (T24/83 bladder carcinoma cells)
ED ₅₀	50% effective dosage
EDTA	ethylenediamine tetra acetic acid disodium salt
EDRF	endothelial derived relaxing factor
EBM-2	endothelial cell basal medium-2
EGM TM -2	endothelial growth medium-2
eNOS	endothelial nitric oxide synthase
Eq.	equation
Eqs.	equations
f	femto
F	permeability ratio
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FDA	food and drug association

FGF2	fibroblast growth factor-2
Fig.	figure
Fig's.	figures
FMN	flavin mononucleotide
g	gram
GFR	glomerular filtration rate
GIT	gastrointestinal tract
Glu	glutamine
[³ H]	tritiated hydrogen
HCl	hydrochloric acid
HCTZ	hydrochlorothiazide
Hcy	homocysteine
HDL	high density lipoprotein
HEPES	(<i>N</i> -[2-Hydroxyl]piperazine- <i>N</i> ¹ -[2-ethanesulfonic acid])
HHF	hypothalamic hypertensive factor
HIV-1	human immunodeficiency virus-1
HOPE-2	Heart Outcomes Prevention Evaluation-2
HUVEC	human umbilical vein endothelial cells
iNOS	induced nitric oxide synthase
<i>I</i> ₅₀	50% inhibition of uptake
K ⁺	potassium
k	kilo
kDa	kilo Dalton
kg	kilogram
<i>K</i> _{<i>D</i>}	diffusion constant
<i>K</i> _{<i>i</i>}	constant of inhibitor
<i>K</i> _{<i>ia</i>}	constant of inhibitor <i>a</i>
<i>K</i> _{<i>ib</i>}	constant of inhibitor <i>b</i>
<i>K</i> _{<i>m</i>}	Michaelis constant
<i>K</i> _{<i>m</i>} '	apparent Michaelis constant
<i>K</i> _{<i>mi</i>}	Michaelis constant of inhibitor

L	liter
L-[³ H]arginine	tritiated arginine; L-[2,3,4- ³ H] monohydrochloride arginine
LAT	neutral amino acid transport (y^+L)
LDL	low density lipoprotein
Li ⁺	lithium
L-NAME	<i>N</i> ^G -nitro-L-arginine-methylester
L-NMMA	<i>N</i> -monomethyl-L-arginine
LPS	lipopolysaccharide
μ	micro
M	molar
M199	culture medium 199
m	milli
MBq	Megabecquerel
5-methyl-THF	5-methyltetra hydrofolate
Mg ²⁺	magnesium
mg	milligram
ml	milliliter
mm	millimeter
mM	milliMolar
mol	moles
mRNA	messenger ribonucleic acid
MTHFR	methyltetra hydrofolate reductase
n	nano
N ₂	nitrogen
N ₂ O ₃	dinitrogen trioxide
Na ⁺	sodium
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
nCi	nano Curie
NEM	<i>N</i> -ethylmaleimide
nm	nanometer

nM	nano molar
nmol	nanomole
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOC-9	MAHMA.NONONate
NORVIT	Norwegian Vitamin Trial
NOS	nitric oxide synthase
ω	omega
O ₂	oxygen
p	pico
PAEC	porcine aortic endothelial cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECy5	phycoerytherin cyan 5
Pen	penicillin
PHM5	glomerular epithelium monoclonal antibody and anti-human endothelium
RDA	recommended daily allowance
rHcy	reduced homocysteine
ROS	reactive oxygen species
S	substrate
[S]	substrate concentration
SEM	standard error of the mean
-SH	sulfhydryl group
SHR	spontaneously hypertensive rats
Strep	streptomycin
SOD	super oxide dismutase
T _{1/2}	half-life
TCA	trichloroacetic acid
tHcy	total homocysteine

tPA	tissue-type plasminogen activator
UEA-I	Ulex europaeus-I
v	rate
VISP	Vitamin Intervention of Stroke Prevention
V_{max}	maximum velocity
V_{max}'	apparent maximum velocity
V_{maxa}	maximum velocity of inhibitor a
V_{maxb}	maximum velocity of inhibitor b
vs	versus
VSMC	vascular smooth muscle cells
WKY rats	Wistar-Kyoto rats

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