CHAPTER 1

General introduction

The prevalence of cardiovascular disease is high not only worldwide but also in South Africa. Indeed, 17% of all deaths in South Africa in 2000 were caused by cardiovascular disease (Bradshaw *et al.*, 2003). Moreover, in South Africa, cardiovascular disease is the leading cause of death in those over the age of 45 years (Gaziano; 2008). Importantly, hypertension remains one of the major risk factors for cardiovascular disease. Hence, improvement in therapeutic approaches to hypertension should be sought. In this regard a better understanding of the mechanisms of hypertension is required. One of the potential causes of hypertension is endothelial dysfunction, which is associated with a decreased production of nitric oxide (NO). Although a number of studies have assessed potential mechanisms responsible for the reduced NO production, questions still remain unanswered. Possible mechanistic factors include increased reactive oxygen species (ROS), increased concentrations of homocysteine, or a decrease in arginine concentrations, and hence reduced NO production.

Endothelial dysfunction in hypertension and cardiovascular disease is attributable to decreased production and/or availability of NO. Nitric oxide is the main endothelial derived relaxing factor (EDRF) responsible for vasodilation and subsequent decreases in blood pressure. In this regard, although increases in homocysteine were previously thought to play a role in hypertension, clinical trials to reduce homocysteine by supplementing the diet with vitamins (eg. vitamin $B_{6/12}$/folate see section 1.3.6) have failed to show beneficial effects on cardiovascular events. A number of mechanisms have been proposed by which hyperhomocysteinaemia contributes to endothelial dysfunction, these will be discussed. Importantly, arginine and homocystine (the free oxidized homocysteine dimer), share the same membrane transporter in the kidney. Hence it has been asserted that increased homocystine may decrease intracellular arginine, the precursor of NO. However, the role of arginine transporters in the regulation of NO synthesis has not been fully established. Moreover, although various anti-hypertensive agents [particularly angiotensin-converting enzyme inhibitors (ACEI's)] have been known to decrease oxidative stress and increase NO

availability (independent of blood pressure), the mechanisms of these effects are not clear. Whether ACEI's have an effect on the membrane transport of arginine and hence the production of NO, is not known.

Hence, the aim of my thesis was to elucidate the role of the endothelial cell membrane transporters of arginine and homocystine on the production of NO. Therefore in this chapter of my thesis I will first discuss the role of the endothelium and its dysfunction in hypertension. The effect and transport of exogenous arginine and homocystine in the human endothelial cell on the subsequent production of NO will be discussed next. The past and present understanding of the membrane transporters responsible for cationic amino acid transport (both arginine and homocysteine are cationic amino acids) will then be discussed. Last I will discuss the effects of some antihypertensive drugs on the transport of arginine and NO production.

1.1 The endothelium

The vascular endothelium consists of a monolayer (Vane *et al*., 1990) of about 1 - 6 x 10^{13} endothelial cells forming an organ of almost 1kg (Sumpio *et al.,* 2002). The endothelium not only functions as a permeability barrier between the vasculature and the blood, but also as a paracrine and endocrine organ involved in immune responses, coagulation, growth regulation and extracellular matrix production (Sumpio *et al.,* 2002). Importantly, the endothelium modulates blood flow (Sumpio *et al.,* 2002), vascular tone (Palmer *et al.,* 1987; Vane *et al*., 1990; Sumpio *et al.,* 2002) and prevents atherosclerosis and thrombosis (Vane *et al*., 1990). The endothelium is semi-permeable and controls the transfer of small as well as large molecules between the intra-vascular space and the interstitium. Under steady state conditions the endothelium partially maintains a non-thrombogenic blood-tissue interface by preventing thrombosis and platelet adherence, promoting thrombolysis and blood flow and maintaining vascular tone. In contrast, activation of the endothelium by stimuli such as thrombin or histamine renders the endothelium pro-thrombotic, pro-proliferative and vasoconstricting. An activated endothelium leads to pathological processes, including hypertension, congestive

heart failure (CHF), primary pulmonary hypertension, atherosclerosis, allograft vasculopathy, sepsis and inflammation (Sumpio *et al.*, 2002).

1.1.1 Endothelial function

1.1.1.1 Normal endothelial function

Healthy endothelial cells produce NO from arginine via reaction with the enzyme endothelial nitric oxide synthase (eNOS) (Palmer *et al.*, 1987). Increased arginine uptake by endothelial cells leads to reduced arterial pressure through a direct alteration of vascular resistance (Rajapakse & Mattson; 2009). In addition to regulating vascular tone (Palmer *et al.*, 1987; Chin-Dusting *et al.*, 2007), NO has potent anti-atherogenic and anti-platelet aggregation properties (Palmer *et al.*, 1987). Futhermore, the healthy vascular endothelium has a major role in maintaining the integrity of blood vessels, regulating vascular structure and atheroprotection (Chin-Dusting *et al.*, 2007). Indeed increased extracellular arginine concentration is reported to enhance NO production (Deng *et al.*, 1995; Rajapakse & Mattson; 2009) which causes vasorelaxation (Deng *et al.*, 1995).

1.1.1.2 Role of endothelial dysfunction in hypertension

In the healthy endothelium, arginine is the substrate for vascular NO production (Kakoki *et al.*, 2002 and 2004). A transient decrease in the availability of NO promotes the release of tissue-type plasminogen activator (tPA) which in turn produces endothelium-derived hyperpolarizing factor. The endothelium-derived hyperpolarizing factor causes hyperpolarization in vascular smooth muscle cells (VSMC) and thus induces vasodilation. In short, in a healthy endothelium, tPA promotes a rapid compensatory mechanism when NO availability is transiently decreased. In patients with primary hypertension, endothelial dysfunction and impaired NO availability or production are possible causes of hypertension (Giannarelli *et al.*, 2009) and target organ damage (Portaluppi *et al.*, 2004). In addition, endothelium-derived hyperpolarizing factor is an ineffective compensatory mechanism for chronically decreased NO availability. Together with endothelial dysfunction, structural and

functional derangements of smooth muscle cells in the vascular wall impair vascular tone, which contributes to the development of hypertension (Rodrigo *et al.,* 2003).

Age affects endothelial function in the same manner as primary hypertension. Moreover, age amplifies the effect of primary hypertension on NO availability (Taddei *et al.,* 2001). However, endothelial dysfunction due to a deficiency of NO is delayed in aging premenopausal women due to the stimulatory effect of oestrogen on NO production. Although NO production is likely to be higher in premenopausal women than in men, it declines after menopause as oestrogen concentrations decrease. As endothelial dysfunction promotes cardiovascular disease and enhances the progression of kidney disease, the more robust NO system in premenopausal women could in part explain the slower rate of progression of kidney disease (Baylis; 2008b) and the cardioprotective effects observed in premenopausal women. Moreover, the degree of impairment of endothelial dysfunction has been shown to be a predictor of future cardiovascular events (Schächinger *et al.,* 2000).

1.1.1.3 Mechanisms of endothelial dysfunction

There is currently controversy as to whether endothelial dysfunction is a primary cause of hypertension, or a consequence of prolonged blood pressure elevation. More importantly, impaired endothelium dependent vascular relaxation in primary hypertension is often not restored by clinically effective (normalization of blood pressure) antihypertensive therapy. Therefore, it has been suggested that endothelial dysfunction becomes irreversible once the hypertensive process is established (Panza, Quyyumi *et al.,* 1993). Hence an understanding of the mechanisms responsible for endothelial dysfunction is of the utmost importance in order to establish effective strategies of prevention.

"Although hypertension has been investigated for a long time, its genesis and exact mechanisms remain elusive" (Mendes-Ribeiro & Brunini; 2004). The high prevalence of cardiovascular disease and the high costs of treatment emphasize the need to address prevention in particular, but also the possible development of alternative treatments for

hypertension. In this regard, the mechanisms of the etiologies of hypertension such as endothelial dysfunction, warrant investigation.

In normal healthy individuals a dose dependent decrease in forearm vascular resistance and an increase in forearm blood flow occur in response to acetylcholine infusion (Panza, Casino, Badar *et al.,* 1993). However, the vasodilatory response to acetylcholine is reduced in patients with primary hypertension (Panza, Casino, Kilcoyne *et al.,* 1993). The decreased vasodilatory response in hypertensive patients is associated with decreased NO release by endothelial cells in response to acetylcholine (Calver *et al.,* 1992). What is responsible for the decreased endothelial response to acetylcholine? As arginine is the precursor of NO, changes in blood flow in response to arginine infusion have been investigated. In normotensive control subjects, Panza, Casino, Badar *et al.,* (1993) observed enhanced increases in forearm blood flow in response to an infusion of acetylcholine, followed by as little as 20μmol min^{-1} arginine. However, no change in blood flow was observed in patients with primary hypertension (Panza, Casino, Badar *et al.,* 1993). Futhermore, infusion of N^G-monomethyl-L-arginine (L-NMMA), an arginine analogue, produced a significantly greater decrease in the vasodilatory response and hence blood flow in control subjects than in primary hypertensive patients. Indeed, L-NMMA had no significant effect on the vasodilatory response in hypertensive patients (Panza, Casino, Kilcoyne *et al.,* 1993). However when using carbachol, a muscarinic agonist; nitroprusside, an endothelium independent vasodilator, (Panza, Quyyumi *et al.,* 1993); and isoproterenol, an adrenergic agonist, no difference was found in the forearm vasodilatory response between normotensive and essential hypertensive patients (Cockcroft *et al.,* 1994). Forearm vasculature responsiveness is therefore not always impaired in patients with primary hypertension (Cockcroft *et al.,* 1994). In particular, the vasodilatory response in patients with primary hypertension is only impaired in response to endothelium-dependent vasodilators (acetylcholine). These findings therefore suggest that in patients with primary hypertension there is a defect in the endothelium-derived NO and consequently an increase in vascular resistance systems (Panza, Casino, Kilcoyne *et al.,* 1993). What are the mechanisms for the impaired endothelial-derived NO production?

Some studies have shown a positive association between homocysteine concentration and systolic and diastolic blood pressures (Mudd *et al.*, 1985; Araki *et al.*, 1989; Malinow *et al.*, 1995). In addition homocysteinaemia may be atherogenic although the mechanism is as yet unknown (Sutton-Tyrrell *et al.*, 1997). High homocysteine concentrations may be related to impaired endothelial function since homocysteinaemia decreases vasodilation by NO, increases oxidative stress which is damaging to the endothelium, stimulates VSMC proliferation and changes the elasticity of the vasculature (Rodrigo *et al.*, 2003). Furthermore, oxidative inactivation of NO and dysfunctioning of receptors related to NO signalling could also cause endothelial dysfunction (Goumas *et al.*, 2001). As arginine is the precursor of NO, endothelial dysfunction may also be due to arginine deficiency which is limiting to NO production (Goumas *et al.*, 2001). Since homocystine (the free oxidized homocysteine dimer) and arginine are transported by the same cell membrane transporters in the epithelium of the kidney (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982), homocysteinaemia could lead to decreased intracellular arginine concentrations. Other potential causes of endothelial dysfunction are increased arginase activity, which degrades arginine to ornithine and urea (Buga *et al*., 1996); decreased conversion of citrulline to arginine (Hecker, Mitchell, Harris *et al.*, 1990); glutamine inhibition of eNOS (Taddei *et al*., 1995) and increased concentrations of the arginine analogue, asymmetric dimethyl L-arginine (ADMA) (Vallance *et al.*, 1992, Miyazaki *et al.*, 1999).

Another possible mechanism for endothelial dysfunction might involve the hypothalamus. In 1997 an endogenous nitric oxide synthase (NOS) inhibitor, hypothalamic hypertensive factor (HHF), was found in the spontaneously hypertensive rat (SHR) hypothalamus. The most striking effect of HHF is an increase in the conversion of arginine to citrulline due to the inhibitory effect of HHF on platelet-derived NOS. Hence an increase in HHF could possibly cause a rise in arterial blood pressure (Morris *et al.*, 1997). It has been suggested by gene studies (Huang *et al.*, 1995) and by NOS inhibitor studies (Marescau *et al.*, 1992) that it is not the neuronal isoform of NOS (nNOS) that is concerned with blood pressure, but rather the endothelial NOS (eNOS) isoform. Therefore, it was postulated that the inhibition of hypothalamic eNOS by HHF is directly involved in the pathogenesis and development of hypertension. However, HHF had no effect on eNOS obtained from bovine aorta (Morris *et*

al., 1997). Hence, the effects of HHF on endothelial function may be mediated through specificity for another isoform of NOS or by another mechanism such as tissue-specific modulation of NOS activity (Morris *et al.*, 1997). Before discussing any of these mechanisms further, I would like to discuss in detail the role of NO in hypertension.

1.2 Role of nitric oxide in hypertension

1.2.1 Characteristics of nitric oxide

Nitric oxide is rapidly released from endothelial cells into the vascular lumen as well as into VSMC (Chin-Dusting *et al.,* 2007). Nitric oxide has an unpaired electron and is thus a free radical and therefore highly active. It has a 1 - 2 second half-life upon contact with tissue as it is rapidly oxidised. In saline, without any form of tissue contact, the half-life is approximately 30 seconds. The molecule rapidly diffuses across all membranes down a concentration gradient (Lancaster; 1994).

1.2.2 Production of nitric oxide

The endothelium is subjected to mechanical forces exerted by pulsatile blood flow. These mechanical forces modulate NO production via shear stress-activated signalling pathways (Boulanger; 1999; Posch *et al.*, 1999; Balligand *et al.*, 2009). Pulsatile shear stress induces rapid elevation of eNOS phosphorylation that peaks at 20 minutes and coincides with the acute phase of NO production in adapted bovine fetoplacental artery endothelial cells (Li *et al.*, 2004). Pulsatile shear stress on bovine fetoplacental artery endothelial cells was also found to elevate eNOS protein concentrations while prolonging the half-life of eNOS messenger ribonucleic acid (mRNA) (Li *et al.*, 2004). The production of NO by endothelial cells in response to flow-dependent shear forces is a fundamental mechanism for regulating vascular tone, peripheral resistance and tissue perfusion (Balligand *et al.*, 2009). Indeed endothelial flow- or shear stress is the most potent stimulus for NO production *in vivo*, leading to vasodilation (Li *et al.*, 2003). The eNOS isoform is most abundantly expressed in cardiovascular tissues and particularly in the endothelium. Endothelial NOS is both acutely

and chronically regulated by shear forces. Together these facts place NO at the centre of homeostatic regulation in cardiovascular physiology (Balligand *et al.,* 2009).

1.2.3 Synthesis of nitric oxide

Nitric oxide is synthesized from the semi-essential amino acid arginine by the complex enzyme eNOS (Knowles & Moncada; 1992). The synthesis of NO is influenced by factors such as arginine availability and cellular amino acid transport. Controversy exists as to whether an eNOS genetic polymorphism could be involved in the cause of primary hypertension. A study on normotensive and hypertensive individuals showed a significant association of the Glu298Asp missense variant in intron 4 of eNOS with primary hypertension (Miyamoto *et al.,* 1998), while a later large study reported no association between the Glu298Asp and hypertension (Candy *et al.,* 2009). Yet, this single nucleotide polymorphism in the eNOS gene coding for a substitution of Glu by Asp at position 298 was shown to be associated with a decrease in NO production in endothelial cells subjected to shear forces (Joshi *et al.*, 2007).

The main control of NO concentration is by regulation of NOS activity (De Wardener; 2001). There are three isoforms of NOS, namely: i) the 150kDa neuronal NOS (nNOS) or NOS1, ii) the 135kDa inducible NOS (iNOS) or NOS2 and iii) the 133kDa, 1203 amino acid protein endothelial NOS (eNOS) or NOS3, located at 7q35-7q36 of chromosome 7, which is most abundant in endothelial cells (Balligand *et al.,* 2009). Approximately 5% of the total NOS is eNOS (De Wardener; 2001). A threefold increase in the expression of eNOS mRNA was shown in bovine aortic endothelial cells (BAEC) subjected to oscillatory shear stress (Cai *et al.,* 2004). Small amounts of NO are produced by nNOS and eNOS in most animal cell types while much larger amounts of NO are produced by iNOS upon stimulation with inflammatory cytokines and lipopolysaccharides (LPS) (Wu & Morris; 1998). The endothelial enzyme, eNOS has a Michaelis-Menten constant (K_m) of 2.9μM for arginine (Pollock *et al.,* 1991) which is similar to the brain enzyme nNOS (Nathan; 1992). The maximum specific activity (maximum velocity) (V_{max}) for purified endothelial enzyme based on the conversion of arginine to citrulline is 15nmol (0.015μM) citrulline mg^{-1} protein min^{-1} (Pollock *et al.,* 1991).

This is substantially less than the activity of the brain enzyme (Nathan; 1992). In cultured porcine aortic endothelial cells (PAEC) the K_m of arginine as substrate for NOS was between three and five micromolar and the V_{max} occurred at 100µM arginine, but interestingly, NO production was inhibited at arginine concentrations above 100µM even though the intracellular substrate content was increased (Su *et al.*, 1997). This suggests that even normal arginine concentrations may inhibit NOS activity, possibly through a second binding site for arginine on eNOS which inhibits the catalytic action of the enzyme (Su *et al.*, 1997). (For discussion of the intracellular arginine pools and the arginine paradox, see section 1.4.4).

Citrulline and NO which are products of arginine metabolism, are both synthesized by NOS from arginine in the presence of co-factors and co-substrates such as oxygen**,** calmodulin, calcium, nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and heme (Alderton *et al.*, 2001). Nitric oxide synthase enzyme has consensus binding sites for FMN and FAD as prosthetic groups, as well as a NADPH binding site. There is an absolute requirement for the cofactor BH_4 (Knowles & Moncada; 1992; Alderton *et al.*, 2001) so that insufficient BH_4 results in uncoupling of eNOS which causes endothelial dysfunction leading to hypertension, hyperlipidaemia and atherosclerosis (Toda *et al.*, 2009). In addition NOS may be subject to negative feedback regulation by NO itself (Buga *et al.*, 1993).

1.2.4 Metabolism of nitric oxide

Arginine is the precursor of NO in endothelial cells (Hecker, Sessa *et al.*, 1990), but arginine causes little or no relaxation of freshly isolated vascular preparations (Thomas *et al.*, 1989). Arginine has no effect on cells cultured in normal medium containing 600µM arginine (Palmer *et al.*, 1988) or endothelial cells *in situ* (Gold *et al*., 1989; Thomas *et al.*, 1989). The lack of potentiation of NO via arginine supplementation in normal endothelial cells is probably due to the cells' high endogenous arginine content (Hecker, Mitchell, Harris *et al.*, 1990). Indeed, arginine does potentiate the release of NO from cultured endothelial cells grown in arginine-free medium (Mitchell, Hecker, Änggård *et al.*, 1990). Hence arginine availability may regulate NO production (Mitchell, Hecker, Vane; 1990).

The effects of NO appear to be dependent on and are attenuated by the presence of thiols within the endothelial cell. The nitrosothiols may act as a store of NO. Endogenous NO has been shown to *S*-nitrosylate high molecular weight thiol-containing proteins (Stamler *et al.*, 1992) such as *S*-nitroso-cysteine. *S*-nitroso-cysteine has similar characteristics to NO (Myers *et al.*, 1990), but a very short half-life (Ramdev *et al.*, 1993), and the biological generation has not been demonstrated (Radomski *et al.*, 1992). Glutathione is an abundant thiol (Meister & Anderson; 1983) which prolongs the half-life and the biological activity of NO (Ramdev *et al.*, 1993; Radomski *et al.*, 1992).

Short exposure (less than 15 minutes) of endothelial cells to homocysteine stimulates NO secretion and results in the formation of *S*-nitrosohomocysteine which is a potent platelet anti-aggregator as well as a vasodilator agent. Effects of NO are mediated through cyclic-guanosine monophosphate (cGMP). However, prolonged exposure of endothelial cells to homocysteine results in impaired NO responses as oxidation of homocysteine results in formation of homocysteine thiolactone, which does not react with NO to form *S*-nitrosohomocysteine. This could account for the endothelial cell toxicity of thiolactone. *S*-nitrosohomocysteine does not result in hydrogen peroxide formation as homocysteine does. Results suggest that due to reformation of *S*-nitrosothiol, toxic effects of homocysteine can be attenuated in the short term. Over longer periods of time, however the adverse vascular properties of homocysteine may result from the inability to sustain *S*-nitrosothiol formation which results in decreasing formation of NO. Indeed, endothelial cells become dysfunctional when homocysteine concentrations are elevated for a prolonged time period (Stamler *et al.*, 1993).

1.2.5 Functions of nitric oxide

1.2.5.1 General functions of nitric oxide

Nitric oxide is a widespread signalling molecule which participates in most cellular and organ functions in the body (Ignarro *et al.*, 1999). Importantly, NO is the major EDRF (Ignarro *et al* 1999) and hence mainly responsible for vasodilation. In addition NO plays a major role in

regulating the binding and release of oxygen to haemoglobin, controls the oxygen supply to the mitochondria, kills parasitic organisms, virus-infected cells and tumour cells, and stimulates new mitochondria production (Chin-Dusting *et al*; 2007). Nitric oxide is also a neurotransmitter, plays a role in immunological reactions (Ignarro *et al.,* 1999; Chin-Dusting *et al.,* 2007), and regulates cardiac contractility and blood pressure (Chin-Dusting *et al*; 2007).

Nitric oxide formed by eNOS and nNOS causes vasodilation and hypotension, increased blood flow and has many beneficial actions as a powerful antioxidant (Toda *et al.*, 2009). Nitric oxide, produced in response to flow-dependent shear forces applied to the surface of vascular endothelial cells (Balligand *et al.,* 2009), regulates vascular tone (Ignarro *et al.*, 1999; Chin-Dusting *et al.,* 2007; Balligand *et al.,* 2009), leukocyte adhesion, anti-coagulation and platelet anti-aggregation (Wu & Meininger; 2000; Chin-Dusting *et al.,* 2007), inhibits platelet adhesion (Toda *et al.,* 2009), superoxide generation, vascular cell adhesion molecules, monocyte chemotactic peptides, release of the vasoconstrictor endothelin I (Wu & Meininger; 2000), decreases VSMC proliferation (Wu & Meininger; 2000; Chin-Dusting *et al.,* 2007; Toda *et al.,* 2009) and regulates peripheral resistance and tissue perfusion (Balligand *et al.*, 2009). Nitric oxide is described as anti-atherogenic (Flynn *et al.,* 2002; Chin-Dusting *et al*; 2007), antiproliferative, anti-thrombotic and as a vasodilator due to many of the abovementioned effects (Flynn *et al*; 2002).

Interactions between NO and oxidative stress due to superoxide anion formation is complex but can be illustrated by the findings that NO prevents oxidative changes of low density lipoprotein (LDL) cholesterol which negatively regulates NO production (Rubbo *et al.,* 2002).

1.2.5.2 Functions of nitric oxide in cardiovascular disease and in hypertension

Endothelial produced arginine affects the vasculature through the production of NO which activates cGMP synthase in VSMC and in turn increases the intracellular cGMP which relaxes VSMC (Li & Försterman; 2000). However, information on factors that regulate NO production in hypertension is limited (Rajapakse & Mattson; 2009). Arginine not only increases the bioavailability of NO by increasing NO production (Kakoki *et al.,* 2004; 2006),

but also reduces the inactivation of NO by superoxides (Wascher *et al.*, 1997). In aortic rings, superoxide dismutase (SOD) increases the half-life of NO, which suggests that basal levels of superoxide are produced together with NO and that superoxide reacts with NO. Results suggest that the endothelium is able to regulate the effects of NO by generating superoxide (Hogg *et al.,* 1992). This provides support for the suggestion that increased superoxide generation may underlie the pathogenesis of hypertension (Nakazono *et al.*, 1991). In this regard, arginine acts as a ROS scavenger and curbs the release of oxygen free radicals from endothelial cells (Wascher *et al.,* 1997). Importantly, arginine can produce NO by reacting with hydrogen peroxide (Nagase *et al.,* 1997). Thus in hypertension, when superoxide concentrations are high, arginine may increase the formation of NO via this non-enzymatic pathway. In hypertension, NOS is uncoupled by insufficient BH_4 which is an absolute cofactor for NOS and subsequently more superoxide than NO is produced. Under these conditions, NO production by arginine via the non-enzymatic route is important. Therefore, arginine can increase NO production in hypertension by a non-conventional pathway (Rajapakse & Mattson; 2009).

1.2.6 Deficiencies of nitric oxide and the consequences thereof

Nitric oxide deficiency develops during the natural aging process of the body. The age-related drop in total body NO is not due to substrate limitations but due to increasing antagonists. With aging, substrate production is preserved, but circulating ADMA, a known NOS inhibitor and arginine analogue increases and thus NO production is decreased. Additionally, age-related oxidative stress leads to NO inactivation as well as the formation of glycosylated end products, which prevent NO from reaching its target VSMC (Baylis; 2008b), possibly resulting in development of hypertension with age.

In healthy humans, the availability of arginine is the rate limiting step in the production of NO (Panza, Casino, Badar *et al.*, 1993). Patients with primary hypertension appear to have reduced (Cadwgan & Benjamin; 1993) or defective (Panza, Casino, Badar *et al.,* 1993; Calver *et al.*, 1992) NO synthesis. Some studies suggest reduced platelet sensitivity to NO in primary hypertension patients (Woods *et al.*, 1993). In addition decreased formation of NO from

platelets in patients with primary hypertension has been shown (Cadwgan & Benjamin; 1993). As such, a possible explanation for impaired vasodilation is reduction of NOS activity in hypertensive patients compared to normotensives.

Many mechanisms are responsible for NO deficiency including limitations of the substrate arginine. Low arginine concentrations can be due to decreased arginine synthesis, increased metabolism by arginase and decreased arginine transport into cells. In addition, a decrease in dimethylarginine dimethylamine hydrolase (DDAH) activity would result in a reduction in the conversion of ADMA to arginine and hence an increase in ADMA concentration and a loss of NO production. Moreover, an increase in ADMA concentration would reduce NOS activity (Baylis; 2008a). Bearing in mind the importance of arginine as a substrate for NO production, various studies have investigated the potential effect of arginine supplementation on endothelial function.

1.2.7 Role of arginine supplementation on endothelial function and nitric oxide production

The hypothesis is that the limitation of arginine availability for NO production plays a crucial role in the development of endothelial dysfunction (Chin-Dusting *et al.,* 2007). However, the age-related decrease in endothelial function in the elderly is not related to decreased arginine uptake (Ahlers *et al.,* 2004). Moreover, administration of exogenous arginine was shown to have no effect on endothelial function in healthy individuals (Creager *et al.,* 1992). Nevertheless, previous studies have reported heterogeneous effects of arginine supplementation on endothelial function in patients with heart failure (Kaye *et al.,* 2000; Kaye *et al.,* 2002; Mendes-Ribeiro *et al.,* 2001; Mendes-Ribeiro & Brunini; 2004; Chin-Dusting *et al.,* 2007; Lichtenstein; 2009). It is possible, that the observed differences in the effect of arginine on endothelial function are due to variations in the duration and the dose of arginine supplementation and disease severity (Chin-Dusting *et al.,* 1996). Hence the effects of arginine and its supplementation will be discussed later (see section 1.4.7).

Furthermore, since homocystine and arginine share the same cell membrane transporters (Cusworth & Gattereau; 1968; Foreman *et al.,* 1982), high homocystine concentrations may lead to decreased uptake of arginine by endothelial cells and thus diminish NO production leading to a dysfunctional endothelium and hypertension. Indeed, a positive association between total homocysteine (tHcy) concentration and blood pressure has been reported (Mudd *et al.,* 1985; Araki *et al.,* 1989; Malinow *et al.,* 1995). Nevertheless, The Framingham Heart Study failed to show a positive association between tHcy and hypertension (Sundström *et al.,* 2003). However, homocysteinaemia has been regarded as a risk factor for atherosclerosis (Malinow *et al.,* 1995; Sutton-Tyrrell *et al.,* 1997; Rodrigo *et al.,* 2003) and hence possibly hypertension. I will therefore discuss the controversies related to the potential role of increased plasma homocysteine in cardiovascular disease (see section 1.3.5).

1.3 Role of homocysteine in cardiovascular disease and hypertension

1.3.1 Introduction to homocysteine

Hyperhomocysteinaemia and homocystinuria are both defined by very high concentrations of tHcy in plasma (Refsum *et al.,* 1998). In homocystinuria large amounts of homocystine are also excreted in the urine (Mudd *et al.,* 2000). When plasma homocysteine concentrations are increased, the terms hyperhomocysteinaemia and homocysteinaemia are used interchangeably for hyperhomocysteinaemia and for tHcy or homocysteine (Mudd *et al.,* 2000). Hyperhomocysteinaemia occurs in five to seven percent of the general population and has been proposed as a risk factor for atherosclerotic and thrombotic disease (Refsum et al., 1998). In a meta-analysis the odds ratio for tHcy as a risk factor for thrombosis was reported as 1.6 [95% CI 1.15-2.22] (Wald et al., 2002), and in a study by Verhoef et al., the odds ratio for atherosclerosis was 1.3 [95% CI 1.0-1.6] for each 1 SD increase of fasting tHcy (5μM), after adjustment for sex, age and other potential confounders (Verhoef et al., 1997). Patients have few symptoms which are often only discovered after a cardiovascular event (Gaustadnes *et al.,* 2000). The proposed mechanisms by which increased plasma concentrations of homocysteine cause accelerated vascular disease include endothelial cell damage, VSMC proliferation, lipid peroxidation, up-regulation of prothrombotic factors XII and V and down regulation of anti-

thrombotic endothelial-derived NO (Mandava & Kent; 2009). Elevated plasma homocysteine concentrations have been shown to be pro-thrombotic and toxic to endothelial cells, hence resulting in decreased NO and increased collagen formation (Elkind *et al.*, 2006). Furthermore, homocysteine reacts with NO to form a *S*-nitrosothiol with very different properties to other thiols such as glutathione. The homocysteine nitrosothiol reacts more rapidly than the glutathione nitrosothiol hence eliciting rapid but not sustained release of NO, resulting ultimately in vasoconstriction (Stamler *et al.*, 1993). Moreover, hyperhomocysteinaemia in animals was associated with endothelial dysfunction accompanied by decreased NO availability, increased susceptibility to arterial thrombosis as well as increased development of atherosclerosis (Lentz; 2005).

Researchers proposed a common transport mechanism for cystine, lysine, arginine and ornithine in the proximal renal tubule (Foreman *et al.*, 1982). Indeed, elevated arginine, lysine, and arginine-glutamate resulted in increased homocystine, cystine and cysteine-homocysteine disulphide renal excretion in rats (Cusworth & Gattereau; 1968). It might be possible that elevated homocysteine concentrations could cause increased renal loss of arginine. Hence, increased homocystine may decrease arginine transport into cells and reduce endothelial cell NO production and thus promote hypertension.

1.3.2 Characteristics of homocysteine

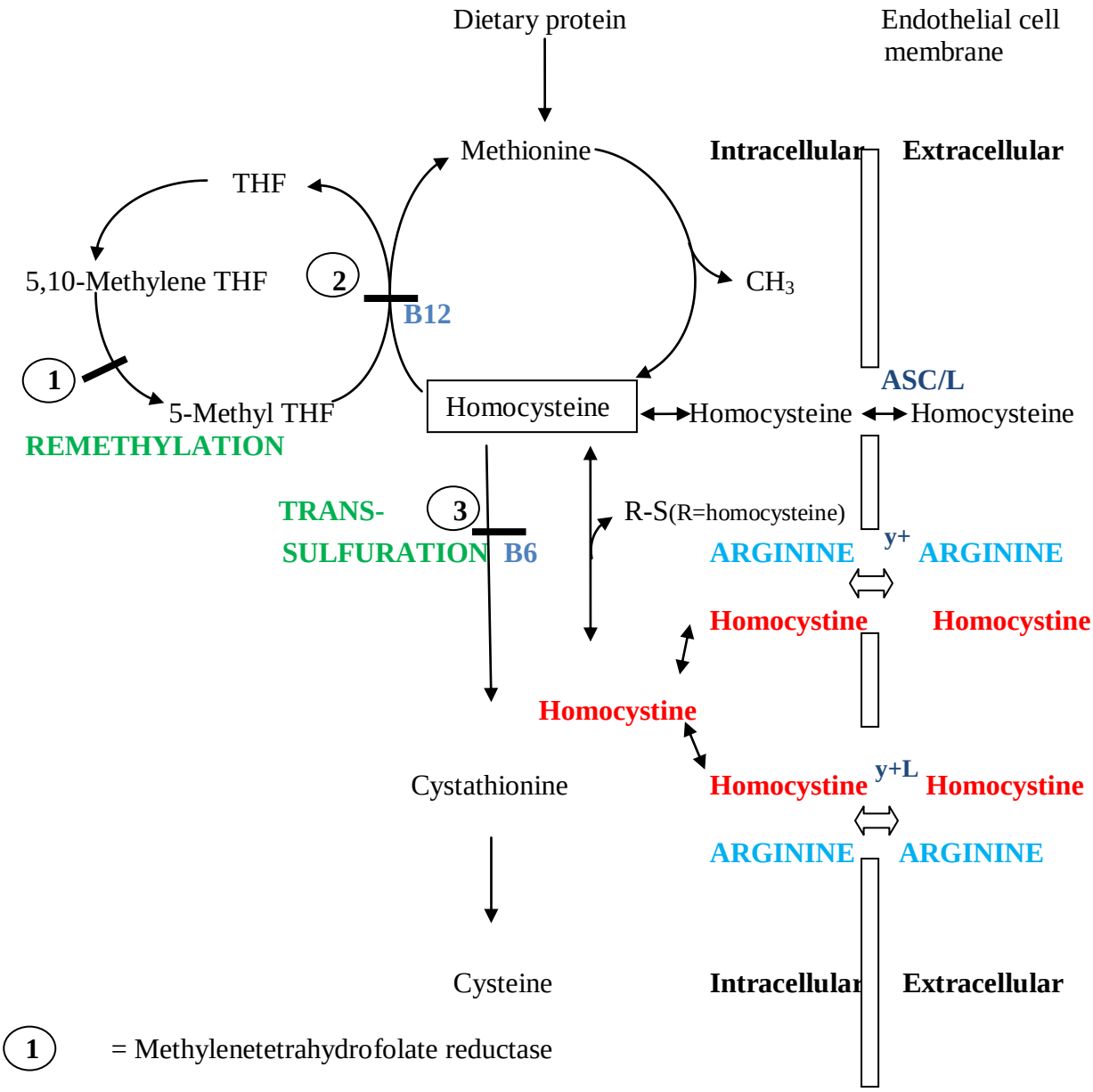
Homocysteine is a sulfur containing amino acid which is structurally similar to cysteine, but contains an extra carbon atom (DuVigneaud; 1952 as cited by McCully; 2007). This sulfur-containing amino acid is an intermediate in the metabolism of cystine and methionine (Ueland & Refsum; 1989; Refsum & Ueland; 1990; Stamm & Reynolds; 1999) and occurs naturally in three distinct forms of which : i) 1.5-4% as the free reduced sulphydryl form of homocysteine (rHcy) (a monomer); ii) 20-30% as the free oxidized homocysteine present as the homocysteine disulphide (the dimer, homocystine) and the mixed disulphide (with cysteine), cysteine-homocysteine and iii) 70-80% as protein bound homocysteine (Mansoor *et al.*, 1992; Andersson *et al.*, 1993; 1995). The sum of all three of these forms is referred to as total homocysteine (tHcy) (Stamm & Reynolds; 1999). Importantly, the amount of the free

homocysteine forms of tHcy is less than $0.1\mu molL^{-1}$ (0.1µM) (Jacobsen; 2001). Since approximately 20-30% of tHcy is dimerised to the disulphide homocystine in solution at physiological pH (Stamm & Reynolds; 1999), and because Dent & Rose; (1951); Cusworth & Gattereau; (1968) and Foreman *et al.,* (1982) showed that homocystine and arginine use the same membrane transporter, I used the dimer homocystine in my studies.

1.3.3 Metabolism of homocysteine (Figure 1.1)

In vertebrates homocysteine can only be formed from *S*-adenosylhomocysteine (AdoHcy) (Refsum & Ueland; 1990), the direct precursor of homocysteine in tissue (Clarke & Banfield; 2001), by the transmethylation process in which *S*-adenosylmethionine is converted to AdoHcy (Lökk; 2003). The hydrolysis of AdoHcy by the enzyme, adenosine-homocysteine hydrolase results in the formation of homocysteine and the purine, adenosine (Ado). This hydrolysis reaction depends on each of the reactants: AdoHcy, homocysteine and Ado. When the homocysteine concentration increases, the reaction will shift towards AdoHcy production and thus lower the free Ado concentration (Riksen *et al.,* 2003). Adenosine has a cardiovascular protective effect (Belardinelli *et al.,* 1989; Mubagwa & Flameng; 2001) and therefore, the lowering of Ado concentrations by an increase in homocysteine concentrations, would decrease the cardiovascular protective effect of Ado (Riksen *et al.,* 2003) and thus increase the cardiovascular risk.

Homocysteine is an end product of the transmethylation of methionine. In addition the concentration of homocysteine depends on the remethylation pathway, forming methionine and the transsulfuration pathway, forming cysteine (Stamm & Reynolds; 1999; Ashfield-Watt *et al.,* 2001). In the remethylation pathway, the enzymes 5,10 methylenetetrahydrofolate reductase (MTHFR) and methionine synthase are involved in the remethylation of homocysteine to methionine (McCully; 1969). The MTHFR enzyme plays a critical role in the regulation of tHcy concentrations by synthesizing 5-methyltetrahydrofolate (5-methyl-THF) which provides the methyl group for the remethylation of homocysteine to methionine (Frosst *et al.,* 1995). 5-Methyltetrahydrofolate acts as a methyl donor for methionine synthase



1 = Methylenetetrahydrofolate reductase

2 = Methionine synthase

3 =Cystathionine β-synthase

THF = Tetrahydrofolate

= possible enzyme deficiency

Figure 1.1: Metabolism and transport of homocysteine and homocystine showing the hypothesized shared transport of arginine and homocystine. **(**Modified from Ashfield-Watt *et al.*, 2001).

while methyl cobalamin serves as a co-factor. In this reaction, 5-methyl-THF is converted to tetrahydrofolate and the methyl group serves to remethylate cobalamin (vitamin B_{12}). The above reactions with methionine synthase link folate and vitamin B_{12} metabolism to the metabolism of homocysteine (Ueland & Refsum; 1989).

In the transsulfuration pathway, the enzyme cystathione-β-synthase (CβS) is responsible for the transsulfuration of homocysteine (McCully; 1969) and hence the formation of cysteine (Stamm & Reynolds; 1999). Deficiencies in any of these three enzymes: MTHFR; methionionine synthase and/or CβS cause homocystinuria with similar vascular pathologies as hyperhomocysteinaemia. This is evidence that homocysteine, which is increased in any of these enzymatic defects, is the toxic factor for developing vascular disease in patients with homocystinuria (McCully; 1969).

A common polymorphism of the MTHFR gene arises from the C substitution to a T at the 677 position resulting in reduced activity of this enzyme (Kang *et al.*, 1988; Goyette *et al.*, 1994) and thus decreased remethylation of homocysteine to methionine. The plasma homocysteine concentrations in TT homozygotes are 25% higher than in the CC 'wild type' individuals. This TT polymorphism occurs in approximately 10 - 15% of Caucasian individuals in the general population (Frosst *et al.*, 1995) and is associated with increased homocysteine concentrations when folate concentrations are low (Harmon *et al.*, 1996; Jacques *et al.*, 1996). However, it is still unclear whether the TT genotype is associated with an increased risk for developing cardiovascular disease (Ashfield-Watt *et al.*, 2001).

In patients with mild homocysteinaemia and premature vascular disease, the 5-methyl-THF-homocysteine methyltransferase activity was normal. Hence it was suggested that the homocysteinaemia in these patients may be caused by a folate deficiency or by a deficiency in CβS activity (Dudman *et al.*, 1993). It was noticed that not only low folate, but also low vitamin B_6 and B_{12} concentrations were associated with increased homocysteine concentrations. As increased homocysteine concentrations were related to increased oxidant stress, impaired endothelial function and increased thrombogenicity (De Bree *et al.*, 2002),

these findings led to the use of vitamin B_6 and B_{12} together with folate to lower homocysteine concentrations.

1.3.4 Plasma concentrations of homocysteine

The normal physiological upper reference concentration for fasting plasma tHcy was determined as 10μmolL^{-1} (10μM) (Ueland *et al.,* 1992; Mudd *et al.,* 2000; Refsum *et al.,* 2004) to 12μmolL^{-1} (12μM) (Ubbink *et al.,* 1995; Rasmussen *et al.,* 1996) or 15μmolL^{-1} (15μM) (Clarke *et al.,* 1991; Graham *et al.,* 1997), in healthy adult males (Wagner & Koury; 2007) with a good vitamin status (Ubbink *et al.,* 1995; Rasmussen *et al.,* 1996). Hyperhomocysteinaemia is defined as a plasma tHcy concentration of greater than 15μM and is denoted as moderate (15 - 30μM), intermediate (30 - 100μM) and severe (>100μM) hyperhomocysteinaemia (Kang *et al.,* 1992 as cited by Refsum *et al.,* 1998). In patients with decreased renal function tHcy concentrations of ≥15μmolL^{-1} (15μM) were noticed in a third of the patients who also had arterial disease (Loncar *et al.,* 2001). Vitamin B_{12} or folate deficiencies cause moderate (>30μmolL^{-1} or 30μM) to severe (100μmolL^{-1} or 100μM) increases in plasma homocysteine concentration (Mudd *et al.,* 2000). In comparison the MTHFR 677TT genotype (Refsum *et al.,* 2004; Andersson *et al.,* 1992) can result in plasma homocysteine concentrations of up to 400μmolL^{-1} (400μM) (Mudd *et al.,* 2000) which cause premature vascular disease (Ueland & Refsum; 1989; Refsum & Ueland; 1990).

Moderate homocysteinaemia with a fasting plasma tHcy of 15 - 30μmolL^{-1} (15 - 30μM) is usually due to a vegetarian diet. Intermediate homocysteinaemia with a fasting plasma tHcy of 30 - 100μmolL^{-1} (30 - 100μM) in otherwise healthy adult individuals is usually a result of vitamin B_{12} or folate deficiency or of renal failure (Refsum *et al.,* 2004). Severe homocysteinaemia with a fasting plasma tHcy of more than 100μmolL^{-1} (100μM) is due to severe vitamin B_{12} deficiency and homocystinuria and should be treated with 20 - 1000μg vitamin B_{12} per day to prevent possible thrombosis (Yap; 2003).

Total Hcy increases by approximately 1μmolL^{-1} (1μM) per decade of age (McCully; 2004) with a 2μmolL^{-1} (2μM) higher level in males than in females at puberty. However, this sex

difference becomes less with increasing age (Jacques *et al.,* 1999). In individuals living in developing countries, tHcy concentrations are high possibly because of dietary effects (Jacques *et al.,* 1999).

1.3.5 Effects of increased plasma homocysteine concentration

Homocysteine has been noted to cause changes in the connective tissues of arteries resulting in fibrosis, calcification and damage to elastic tissue layers (McCully; 2007); hence leading to the formation of plaques and reduced arterial elasticity which in turn may cause primary hypertension (Rodrigo *et al.,* 2003). Homocysteine promotes fibrin deposition, causing thrombosis in artery walls (McCully; 2007), and antagonizes the vasodilatory effects of NO by forming *S*–nitrosohomocysteine which leads to endothelial dysfunction (Stamler *et al.,* 1993). In addition, elevated homocysteine concentrations in asymptomatic individuals may reduce regional systolic heart function (Nasir *et al.,* 2007). These data suggest that increased homocysteine concentration may be an independent risk factor for the development of cardiovascular disease. Indeed, the deleterious effects of homocysteine on endothelial and vascular function together with the effects of increased coagulation provide pathophysiological explanations for the increased cardiovascular risk noted in patients with hyperhomocysteinaemia (Refsum *et al*., 1998). Hence, McCully (1969) proposed the homocysteine theory of atherosclerosis in the general population (McCully; 1969). The development of cardiovascular disease was suggested to be dose dependent with tHcy concentrations of 20μmolL^{-1} (20μM) or greater being an independent risk factor (Refsum *et al*., 1998). However, significant increases in cardiovascular risk were found at tHcy concentrations as low as 13 - 15μmolL^{-1} (13 - 15μM) (Christen *et al.,* 2000). Although no specific cut-off point exists for normal plasma homocysteine, concentrations below 15μmolL^{-1} (15μM) are generally considered normal (Refsum *et al.,* 2004) and increased tHcy is regarded a prognostic marker for increased risk of cardiovascular events or mortality (Ueland *et al.,* 2000).

Even though it is widely accepted that elevated plasma tHcy is an independent risk factor or predictor of vascular disease, no mechanism has been accepted for the pathophysiology (Wagner & Koury; 2007; Mikael *et al.,* 2006; Quinlivan & Gregory; 2006). Proposed

mechanisms by which hyperhomocysteinaemia contribute to endothelial dysfunction and vascular disease include: production of ROS (Heinecke; 2001), induction of apoptosis (Hossain *et al.,* 2003) and reduced expression of the atheroprotective ApoA-1 [which is the major protein component of high density lipoprotein (HDL)], when folate is low (Mikael *et al.,* 2006). Free homocysteine also inhibits production of the vasodilator, endothelin-1 by endothelial cells (Demuth *et al.,* 1999). Another suggestion for the mechanism of action of homocysteine is that through the enzymatic metabolism of homocysteine, homocystine or its mixed disulphide with cysteine, the unstable reactive compound, sulfane sulfur is formed which plays a role in atherogenesis (Toohey; 2001).

Data from retrospective and case-control studies support a role for homocysteine in increasing cardiovascular risk. Ueland *et al.,* (1992) in a review of 21 case-control studies including more than 1500 patients with occlusive vascular disease (cerebrovascular-; peripheral- and cardiovascular-) and more than 1500 control individuals, indicated that in 16 of the 21 studies, the homocysteine concentrations were reported to be mildly (patient 1.13: control 1) though significantly elevated ($p < 0.05$ to $p < 0.001$) in the patients. Data from two other case-control studies and from a retrospective case-control study, suggested that homocysteine was associated with progressive arterial disease (McCully; 1969; De Bree *et al.,* 2002). In a meta-analysis of 27 studies of which three were prospective studies, six were population based case-control studies, five were cross sectional studies and 13 were other case-control studies, it was concluded that homocysteine causes peripheral-, cardio- and cerebrovascular disease (Boushey *et al.,* 1995). Furthermore, the prevalence of hyperhomocysteinaemia reported in patients with cerebral vascular disease was 42%; with peripheral vascular disease was 28% and with cardiovascular disease was 30% (Ueland *et al.,* 1992; Clarke *et al.,* 1991).

Although data from retrospective and case-control studies indicate that increases in plasma homocysteine concentrations are associated with increased risk of cardiovascular diseases (Christen *et al.,* 2000), most prospective studies are ambiguous as to the effects of elevated homocysteine on cardiovascular disease (Stampfer; 2002). Results range from a significant positive association (Stampfer; 2002) to non-significant (Alfthan *et al.,* 1994) and weak associations (Verhoef *et al.,* 1994; Ashfield-Watt *et al.,* 2001) between plasma homocysteine

concentrations and cardiovascular disease. Therefore, it has been suggested that homocysteine concentration is just a marker, rather than a cause of cardiovascular disease (McCully; 2004). Indeed, a three year follow-up study failed to report an association between homocysteine and risk of myocardial infarction (Chasan-Taber *et al.,* 1996), and an 11 - 17 year follow-up study failed to prove an association between homocysteine and coronary heart disease (Evans *et al.,* 1997). Nevertheless, it was found that baseline homocysteine concentrations were increased in patients with coronary heart disease who died within the first five years of follow-up (Evans *et al.,* 1997), and in a general population study mild increases in plasma homocysteine were associated with an increased risk of developing atherosclerosis and venous thrombosis (Ashfield-Watt *et al.,* 2001). Moreover, increased concentrations of homocysteine were associated with a three-fold increase in cardiovascular risk upon a two year follow-up of healthy, well nourished physicians (Stampfer *et al.,* 1992).

Because of the controversies of the relationship between homocysteine and vascular diseases, it has been suggested that homocysteine is a product rather than a cause of vascular disease (Knekt *et al.,* 2001), and homocysteine may be a predictor of late-stage coronary heart disease (Ashfield-Watt *et al.,* 2001) and a predictor of secondary but not primary vascular events (Anderson *et al.,* 2000; Knekt *et al.,* 2001). Time-related changes in homocysteine are important in the estimation of the strength of the association between hyperhomocysteinaemia and cardiovascular disease, and statistical adjustment for regression dilution bias has to be taken into account. By using the baseline homocysteine concentrations in assessing cardiovascular disease, the hyperhomocysteinaemia/cardiovascular risk is underestimated by a third after four years of follow up and by half after ten years. Therefore, analysis not making this regression dilution bias adjustment would underestimate the real association between homocysteine and cardiovascular disease (Ashfield-Watt *et al.,* 2001). This observation could partly account for the controversial findings of homocysteine as a predictor of cardiovascular disease.

In an attempt to resolve the controversies as to the association of tHcy with cardiovascular disease, researchers have investigated the potential role of folate and vitamin B_{12}. Folate and vitamin B_{12} are linked to homocysteine metabolism (see Figure 1.1), in the methylation

pathway. Indeed, plasma homocysteine concentrations were found to be inversely related to folate concentrations in black women with preeclampsia (increased blood pressure developed in pregnancy in women who prior to pregnancy had normal blood pressure) (Patrick *et al.*, 2004). In this regard, it has been suggested that folate might have a direct beneficial effect on endothelial function independent of homocysteine (Ashfield-Watt *et al.,* 2001). Furthermore, homocysteine concentrations were increased in patients with nutritional deficiencies in folate and vitamin B_{12} (McCully; 2007). Despite the suggested association of decreased folate and vitamin B_{12} with increased homocysteine concentrations, trials using either folate or a combination of folate and vitamins B_6 and B_{12} at doses which lower plasma homocysteine concentrations reported no significant effects on cardiovascular events (Davey Smith & Ebrahim; 2005; Bleys *et al.,* 2006).

Although increased plasma tHcy concentrations are still commonly used as a marker of folate and vitamin B_{12} deficiency (Savage *et al.,* 1994; Klee; 2000) and as an independent risk factor for cardiovascular disease (Refsum *et al.*, 1998), it is difficult to prove that increased tHcy is a main cause of cardiovascular disease (Wagner & Koury; 2007). Hence, whether increased tHcy concentration is a cause of vascular risk remains largely unclear.

1.3.6 Intervention to correct hyper/homocysteinaemia and homocystinuria

Bearing in mind the detrimental effects of elevated tHcy discussed above, various interventions to decrease plasma homocysteine concentrations have been investigated.

Deaths from heart attack and vascular disease reached a peak in the 1950's in the United States. The dramatic decrease in cardiovascular deaths in the United States since then is possibly due to the fortification of foods with vitamin B_6 and folate which increase blood folate and decrease tHcy (McCully; 2007). The Framingham Heart Study showed that tHcy concentrations decreased by 15% and blood folate concentrations almost doubled after grains were folate fortified in 1998 in the United States (Jacques *et al.,* 1999). Folate in the form of 5-methyl-THF as a co-substrate and vitamin B_{12} as a co-factor for methionine synthase, are involved in the remethylation process of homocysteine, thereby decreasing plasma tHcy

(Ashfield-Watt *et al.,* 2001). In a study of 161 patients with silent brain infarcts, the mean plasma tHcy level was found to be increased compared to the mean level in normal healthy controls. However, the folate and homocysteine concentrations in silent brain infarct patients showed an inverse relationship only in patients with the MTHFR 677TT genotype. Hence, folate supplementation could reduce the risk for silent brain infarct in these particular individuals (Kim *et al.,* 2003). Folate given to patients with chronic renal insufficiency, decreased plasma tHcy concentrations by 53% ($p < 0.001$) while red cell folate concentrations remained unchanged (Wilken *et al.,* 1988). Furthermore, depletion of tissue folate may cause homocysteinaemia in non-homocysteinuric patients (Kang *et al.,* 1987). Folate alone appears to reduce homocysteine concentrations regardless of the cause of elevated homocysteine concentration (Manson *et al.,* 1993).

In an experimental study of rats on a diet containing homocysteine or homocysteine plus folate, plasma tHcy concentrations and cerebral vascular damage in the homocysteine only group were significantly increased. The plasma tHcy concentrations of the rats fed homocysteine and folate were the same as the concentrations in the control group on a normal diet. These homocysteine concentrations and electron microscopy findings suggest that folate supplementation possibly reduces the detrimental effects of high homocysteine concentrations on the endothelium and reduces cerebrovascular risk (Lee *et al.,* 2005). Hence, it was suggested that folate supplementation could decrease plasma tHcy concentrations. However, the most significant decrease in homocysteine was found in patients given a combination of vitamins B_6, B_{12} and folate (Ubbink *et al.,* 1993).

Treatment with vitamin B_6 and/or folate and vitamin B_{12} from infancy can prevent cardiovascular disease and associated symptoms (Yap *et al.,* 2001; McCully; 2007). Significant inverse correlations between homocysteine and folate, vitamins B_6 and B_{12} have been observed in both young- and elderly healthy individuals (Joosten *et al.,* 1993). A study involving 1160 patients aged between 67 - 96 years showed that plasma homocysteine concentrations were inversely correlated with plasma folate, vitamin B_{12} and pyridoxal-5'-phosphate (vitamin B_6). In two thirds of these cases, inadequate intake of one or more vitamins appeared to contribute to high homocysteine concentrations. Indeed, the

homocysteine concentration was twice as high in asymptomatic vitamin B_{12}-deficient individuals compared to controls (Selhub *et al.,* 1993). These data are supported by a study involving 14916 United States physicians in which homocysteine concentrations correlated inversely with the intake of vitamins $B_{1,}$ $B_{2,}$ $B_{6,}$ $B_{12,}$ niacin, folate, C, E, retinol and with plasma B_6 concentrations and the use of multivitamins (Stampfer *et al.,* 1992).

However, although initial clinical trials suggested coronary artery plaque regression upon homocysteine lowering with folate and vitamin B supplementation, recent clinical trials failed to document beneficial outcomes in patients with atherosclerosis due to homocysteine lowering (Antoniades *et al.,* 2009).

Patients with non-disabling cerebral infarction who took part in the Vitamin Intervention of Stroke Prevention (VISP) trial were given a folate, vitamin $B_{6/12}$ cocktail. Upon a two year follow-up, decreased tHcy concentrations, but no vascular effects of treatment were found (Toole *et al.,* 2004). Furthermore, heart transplant patients who were on folate treatment for one year, showed a decrease in tHcy concentration, but no effect on the coronary arteries (Potena *et al.,* 2005). Patients with myocardial infarcts included in the Norwegian Vitamin (NORVIT) trial were treated with folate with or without vitamins $B_{6/12}$ within seven days of a myocardial infarction, but after three years of treatment, no significant effect was noted on recurrent cardiovascular disease (Bønaa *et al.,* 2006). These results were disappointing however, 80% of patients were receiving statins and 30% ACEI's, hence the mild effects of homocysteine may have been masked by the effects of these medications. Moreover, the simultaneous initiation of homocysteine-lowering treatment together with other drugs had a strong pleiotropic effect which could have covered the possible effect of homocysteine-lowering treatment (Antoniades *et al.,* 2009). The Heart Outcomes Prevention Evaluation-2 (HOPE-2) trial on patients with vascular disease or diabetes, treated with folate and vitamins $B_{6/12}$ for approximately five years, showed no significant improvement on cardiovascular or myocardial infarct deaths (Lonn *et al.,* 2006). Thus, available data do not show folate and vitamin B supplementation benefit in the reduction of cardiovascular disease (Bleys *et al.,* 2006; Refsum & Smith; 2006). Furthermore, the statistical power of this trial was significantly weak for subgroups living in non-folate-fortified areas, therefore, this trial also

failed to show an effect of homocysteine-lowering treatment on cardiovascular risk (Antoniades *et al.,* 2009).

There are a number of additional reasons for the failure of the abovementioned trials, including that the recommended daily allowance (RDA) for folate of 400μg per day has the ability to maximally benefit vascular function. Therefore, if this amount is already present in flour or grains by fortification with folate or by a folate-rich diet, no additional benefit would be observed by any additional folate treatment. Moreover, by administering folate, the methylation of arginine is enhanced (Antoniades *et al.,* 2009) resulting in the formation of arginine residues. Arginine residues increase ADMA concentrations which uncouple eNOS, leading to a NO deficiency with adverse clinical outcomes (Antoniades *et al.,* 2009). Additionally, hypermethylation of the promoter regions of pro-atherogenic genes upregulate a wide range of pro-atherogenic molecules (Antoniades *et al.,* 2009), promoting atherosclerosis. Another possibility is that folate could promote cell proliferation through its role in thymidine synthesis, which could lead to the worsening of atherosclerosis (Antoniades *et al.,* 2009).

Despite data from large clinical trials (VISP; NORVIT and HOPE-2) failing to show beneficial effects of vitamin supplementation on cardiovascular disease (Toole; *et al.,* 2004; Potena *et al.,* 2005; Bønaa *et al.,* 2006), some smaller interventional studies reported decreases in tHcy (Kim *et al.,* 2003; Wilken *et al.,* 1988; Manson *et al.,* 1993; Ubbink *et al.,* 1993) and beneficial effects in cardiovascular disease (Yap *et al.,* 2001; McCully; 2007) upon vitamin supplementation. Therefore, although it has been documented that vitamin supplementation improved prognosis of cardiovascular events in some cases despite persistently high tHcy concentrations (Yap *et al.,* 2000), it seems possible that vitamin supplementation improves vascular prognosis by other mechanisms of action rather than by homocysteine lowering (Ashfield-Watt *et al.,* 2001). Although it is clear from trials and studies that vitamin supplementation does lower tHcy in the young, the aged, in health and in various disease states it is not clear whether it is the lower tHcy concentration or the vitamin supplementation that is beneficial in the prevention or improvement of vascular disease.

It is currently accepted that moderate or severe homocysteinaemia is associated with thromboembolic events and atherothrombosis and should be treated with folate and B vitamins. Yet, it is still unclear whether this treatment improves the clinical situation of individuals with mild homocysteinaemia or is preventative in individuals with tHcy concentrations in the normal range (Antoniades *et al.,* 2009). The controversies in homocysteine lowering by vitamins and the controversial role of homocysteine concentrations in cardiovascular disease has turned researchers attention to the possibilities of arginine supplementation as a means of improving endothelial function and hence alleviating cardiovascular disease and hypertension.

1.4 Role of arginine in cardiovascular disease and hypertension

1.4.1 Characteristics of arginine

Arginine (2-amino-5-guanidinovaleric acid), was identified as the physiological precursor of NO synthesis in animal cells (Palmer *et al.*, 1988). Two arginine stereoisomers namely the L- and the D- type exist. The L- and the D-types of arginine are compounds with the same molecular formula but different structures so that the L-type arginine is a mirror image of the D-type. Previous studies showed that in human skin fibroblasts, hepatoma cells, renal cells and intestinal cells the affinity for D-arginine was approximately 20 times less than for L-arginine (White; 1985). Hence, I used L-arginine in all my experiments and therefore in this thesis the word arginine refers to L-arginine unless specified as D-arginine.

Arginine is not an essential amino acid hence, the endogenously produced arginine is adequate for the metabolic needs of a healthy adult. However, arginine is classified as a conditionally essential or semi-essential amino acid since in stress situations such as excessive heat, presence of ROS, increased mechanical forces (Barbul; 1986), severe trauma (Morris Jr; 2002) and pathological states (Barbul; 1986), which alter endothelial function and hence NO production, dietary intake of exogenous arginine is required (Barbul; 1986; Morris Jr; 2002).

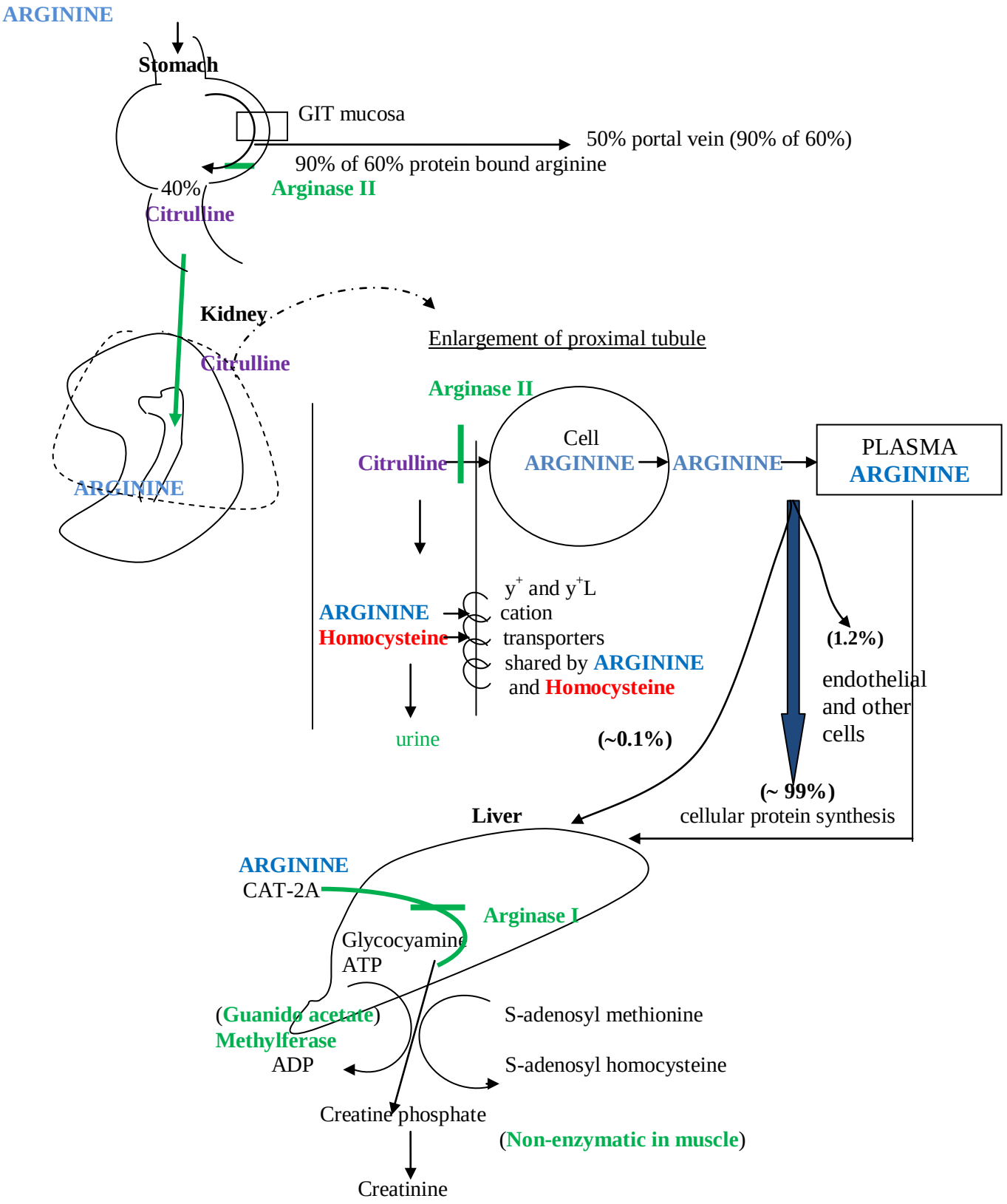


Figure 1.2 Arginine metabolic pathway in the gastro-intestinal tract (GIT) and liver.

1.4.2 **Metabolism of arginine** (Figure 1.2).

Dietary arginine is catabolized by the small intestinal mucosa (Wu; 1998) to produce citrulline which is released into the blood. Little or no citrulline is extracted from blood by the liver during hepatic circulation (Wu; 1998; Morris Jr; 2002) since hepatocytes have a low permeability for citrulline (Levillain *et al.,* 1990; Meijer *et al.,* 1990). Hence, most citrulline remains in the blood during transhepatic transit and is thus available for extrahepatic arginine synthesis (Levillain *et al.,* 1990; Meijer *et al.,* 1990). Citrulline is efficiently taken up from the blood by the proximal renal tubules where it is converted to arginine which is released back into the blood (Wu & Morris; 1998; Morris Jr; 2002). Most of the arginine synthesis in mammals involves this intestinal-renal axis (Wu & Morris; 1998).

In addition to the intestinal-renal axis, a small proportion of arginine is formed in the liver and kidneys, within the urea cycle from the condensation of citrulline and aspartate. This condensation process is a two step reaction, which requires adenosine triphosphate (ATP). In the first reaction, catalysed by arginosuccinate synthase also termed L-citrulline: L-aspartate ligase, adenosine monophosphate (AMP), pyrophosphate and arginosuccinic acid are produced. In the second reaction argininosuccinate: arginine lyase catalyses the cleavage of arginosuccinic acid to form fumaric acid and arginine (Wu & Morris; 1998). Although some arginine is synthesized in the liver (Reyes *et al.,* 1994; Wu & Morris; 1998) it does not contribute significantly to the plasma concentrations of arginine since it is routed to local utilization (Reyes *et al.,* 1994; Wu & Morris; 1998) by arginase I for the urea cycle to produce ornithine and hence is not available for hepatic export.

Importantly, the arginine produced by the proximal kidney cortex tubules is distributed throughout the body (Wu & Morris; 1998). Arginine resynthesized from citrulline, together with kidney arginine synthesis maintains the plasma arginine concentrations (Reyes *et al.,* 1994) between 100 - 200$\mu molL^{-1}$ (Mendes-Ribeiro *et al.,* 2001). In the kidney arginine is synthesized in decreasing amounts along the length of the proximal convoluted tubule (122 ± 15fmol min^{-1} mm^{-1}) to the medullary straight tubule (41 ± 4fmol min^{-1} mm^{-1}). No arginine synthesis has been observed in the glomerulus or other parts of the nephron. The amount of

arginine synthesized is proportional to the concentration of the citrulline precursor. In the proximal convoluted tubule, citrulline is taken up by the endothelial cells and is converted into arginine. Normal circulating citrulline concentrations are approximately 0.1mM (100μM) and due to synthesis by enterocytes, citrulline concentrations increase after a protein meal. Increased citrulline after a protein meal results in enhanced arginine synthesis in the postprandial hours. The increased arginine concentrations result in vasodilation of the afferent arteriole resulting in increased glomerular filtration rate (GFR) (Levillain *et al.,* 1990).

Newly synthesized arginine diffuses into the blood and is available for protein synthesis and metabolism. The arginine may form guanidine derivatives within the kidney. In the cortical straight tubules, 40% of the arginine formed is converted into urea and ornithine, while this conversion amounts to approximately 64% in the medullary part (pars recta). The urea formed serves to concentrate urine and the ornithine may be used to form polyamines (Levillain *et al.,* 1990). Importantly, the remainder of arginine (60% from cortical straight tubules and 36% from medullary part) is available for other functions including the formation of NO.

Arginine is degraded by arginase enzymes. Arginase I is a cytosolic enzyme which is highly expressed in the liver as a component of the urea cycle as well as in some other tissues. Type II arginase is a mitochondrial enzyme expressed in much lower concentrations in extrahepatic tissues including the kidney, brain, small intestine, endothelial cells, mammary gland and macrophages (Wu & Morris; 1998). As both arginase and NOS enzymes use arginine as a substrate, arginase I and II also play a role in the regulation of NO synthesis in mammalian cells (Ignarro *et al.,* 2001; Wei *et al.,* 2001; Li *et al.,* 2001; Li, Meininger *et al.,* 2002). Approximately 99% of plasma arginine is used for protein synthesis while only about 1.2% of plasma arginine is associated with NO production and a very small percentage is metabolised in the liver. The 1.2% of plasma arginine contributes to approximately 60% of arginine precursor available for NO synthesis in endothelial cells (Castillo *et al.,* 1996; Chin-Dusting *et al*; 2007).

Arginine is transported into the liver by the cationic amino acid transporter -2A (CAT-2A) and is degraded by hepatic arginase I (Closs, Albritton *et al.,* 1993). The arginine degraded by

arginase I is converted to creatine phosphate which is in turn converted non-enzymatically to creatinine in the muscle. Hence it is possible that hepatic transport regulates plasma arginine concentrations. The mRNA for CAT-2A is the only CAT isoform mRNA expressed in the liver (Kitiyakara *et al.,* 2001). The CAT-2A transporter has a low affinity for arginine and thus limits the hepatic uptake and clearance of arginine which in turn protects the plasma arginine concentrations (White & Christensen; 1982a). The fact that CAT-2A has a low affinity for arginine, predicts that uptake of arginine by the liver is dependent on the expression of the transporter. It is therefore suggested that circulating concentrations of arginine are tightly controlled (Kitiyakara *et al.,* 2001).

1.4.3 Plasma concentrations of arginine

The concentration of arginine in human plasma ranges from 80 - 120μM (Mendes-Ribeiro *et al.,* 2001). However concentrations as high as 200μM have been reported under normal physiological conditions (Wu & Morris; 1998). Although administration of arginine increases NO production, when only 50μM arginine was administered, endothelial cells failed to elicit NO production (Chin-Dusting *et al.,* 2007), which suggests that extracellular arginine concentrations are rate limiting for the production of NO (Mitchell, Hecker, Vane; 1990). Hence, deficiencies in plasma arginine concentrations may be responsible for endothelial dysfunction. Indeed, in young pre-hypersensitive SHR, plasma arginine concentrations were found to be decreased (Jones; 1988) compared to Wistar-Kyoto rats (WKY); whereas urinary arginine concentrations were similar in the two groups. In addition to decreased plasma arginine, lysine and ornithine were also decreased in SHR (Jones; 1988). As these amino acids share a common transporter (Cusworth & Gattereau; 1968; Foreman *et al.,* 1982), a possible reason for the decreased plasma concentrations may be a defect in the common transporter. Alternatively, the high homocystine concentrations may be responsible for the decreased arginine concentrations, as renal reuptake of arginine is decreased by homocystine (Foreman *et al.,* 1982). However, as discussed in sections 1.4.1 and 1.4.2, in healthy subjects arginine is not an essential amino acid as it can be produced endogenously from citrulline.

1.4.3.1 Endogenously produced arginine

Arginine is produced endogenously from citrulline in various cells including endothelial cells. Indeed, in order to continuously form NO, cultured endothelial cells maintain their arginine concentrations by generating endogenous arginine from citrulline (Hecker, Mitchell, Harris *et al.*, 1990; Mitchell, Hecker, Änggård *et al.*, 1990).

However, when PAEC were cultured for 24 hours in arginine free medium, the intracellular (endogenous) arginine was decreased by 50%. In contrast, when human umbilical vein endothelial cells (HUVEC) were cultured for 24 hours in arginine depleted medium, no change in intracellular arginine concentrations were noticed (Morgan & Baydoun; 1994). Therefore, in some vascular endothelial cell types, exogenous arginine is essential to maintain the intracellular arginine concentrations and hence the production of NO. Under these circumstances the efficiency of the transport of exogenous arginine into the endothelial cells may influence the intracellular arginine concentrations (see section 1.5.8.2). Hence, exogenous (plasma) arginine concentrations may impact on endogenous (intracellular) arginine concentrations. However, whether both plasma and intracellular arginine concentrations influence NO production warrants discussion.

1.4.4 The arginine paradox

Intracellular (endogenous) arginine concentrations range between 100 - 2000μM which are sufficient to saturate the eNOS enzyme and produce NO as the K_m of eNOS for arginine ranges from approximately 2.9μM - 10μM. The saturation of eNOS implies that eNOS would be unlikely to respond to changes in extracellular arginine concentration (Harrison; 1997). However, extracellular (exogenous) arginine concentrations from 0.1 - 10 000mmol L^{-1} (0.1 - 10 000μM) have been shown to further increase NO production in spite of the saturation of the eNOS enzyme with intracellular arginine. This phenomenon is called the arginine paradox (Cooke *et al*; 1991; Harrison; 1997; Li & Försterman; 2000). It was speculated that some of the intracellular arginine is located in a second arginine pool (pool II) which is less available for transport but is normally accessable to eNOS and this could underlie the arginine paradox;

however, impaired access to pool II by eNOS could explain how endothelial NO production can be increased by extracellular arginine.

The arginine 'paradox' may also be explained by the possibility of the colocalization of eNOS and the CAT-1 arginine transporter in caveolae (membrane associated vesicles). Extracellular arginine is readily available to CAT-1 and because eNOS and CAT-1 are located closely together, eNOS might prefer arginine transported from the extracellular fluid by CAT-1 (McDonald, Zharikov *et al.*, 1997; Rajapakse & Mattson; 2009). In addition, intracellular arginine compartementalization, interaction of arginine with glutamine, eNOS dimerization alterations and the competitive inhibition of eNOS by endogenous inhibitors such as ADMA (Maxwell & Cooke; 1998) may play a role. Assymetric dimethyl L-arginine concentrations are often increased in hypertension, hypercholesterolaemia, hypertriglyceridaemia, insulin resistance, renal failure, type II diabetes mellitus and coronary syndrome X. Although ADMA may compete with arginine for transport by the cationic transporter system, it is unclear whether this competition occurs in physiological and/or pathophysiological circumstances. However, there are both experimental and clinical evidence that small changes in ADMA concentrations change NO production, vascular tone and vascular resistance significantly (Chin-Dusting *et al*; 2007).

A futher explanation for the arginine 'paradox' is that the arginase enzyme is responsible for converting arginine to ornithine and urea and hence is important in regulating intracellular arginine concentrations *in vivo* (Nagase *et al.*, 1997). Vascular smooth muscle cells exclusively express arginase I whereas endothelial cells mostly express arginase II (Durante *et al.*, 2001). Both arginase I and II regulate NO synthesis by using arginine as a substrate (Ignarro *et al.*, 2001; Wei *et al.*, 2001; Li *et al.*, 2001; Li, Meininger *et al.*, 2002). *N*-hydroxy-L-arginine, an intermediate in the NOS pathway, is an inhibitor of arginase. Indeed, arginase activation can decrease intracellular arginine concentrations which can result in NOS dysfunction and thus impaired NO production (Tousoulis; 2002). Hence, both exogenous and endogenous arginine concentrations are important in maintaining normal endothelial function and NO production.

1.4.5 Functions of arginine

Biochemically, arginine is the precursor to the endogenous relaxing factor, NO, as well as the precursor to the polyamines: putrescine, spermidine and spermine. Arginine also plays a pivitol role in protein metabolism as it has roles in protein synthesis, is a precursor for creatine and other guanidine derivatives, and is important in nitrogen balance as arginine is converted to urea and ornithine by arginase (Wu & Morris; 1998; Wu *et al.*, 2009). Arginine also has other NO-independent properties, such as regulating blood and intracellular pH and has an effect on the depolarization of endothelial membranes (Tousoulis; 2002). Physiologically, arginine has antihypertensive properties by being the precursor of NO which causes vasodilation. Arginine also has antioxidant properties since arginine acts as a scavenger of ROS (Wascher *et al.,* 1997), and stabilizes all isoforms of NOS, thereby inhibiting superoxide formation (Alderton *et al.,* 2001). Arginine influences the viscosity of blood and the coagulation/fibrinolysis system, and it also affects glucose- , lipid- and protein metabolism (Tousoulis; 2002). Hence the administration of arginine reverses endothelial dysfunction, enhances wound healing and improves cardiovascular-, reproductive-, pulmonary-, renal-, digestive- and immune functions (Flynn *et al.*, 2002; Wu, Meininger, Knabe *et al.*, 2000). Apart from its endothelial and antihypertensive effects, arginine alters the inflammatory- and thrombotic mechanisms in patients with atherosclerosis (Tousoulis; 2002). Although arginine administration improves endothelial function in patients with hypercholesterolaemia, hypertension and smoking, it has no effect on endothelial function in patients with diabetes mellitus (Tousoulis; 2002). Bearing in mind that arginine has beneficial effects on the cardiovascular system, it is important to discuss the consequences of arginine deficiency.

1.4.6 Deficiency of arginine

The average American adult diet with red meat as a source of arginine contains approximately 5.4g arginine per day (Visek; 1986), of which about 40% is degraded to citrulline which is converted back to arginine in the GIT (gastrointestinal tract) by the high arginase II activity in the small intestinal mucosa. The remainder which is protein bound (60%) enters the portal vein (Wu; 1998; Castillo *et al*., 1993). The digestibility of protein bound arginine is 90% in

adults and neonates (Wu; 1998) and hence only about 50% of dietary arginine enters the systemic circulation via the portal vein (see figure 1.2). Therefore, oral administration of supplemental arginine is less effective than intravenously administered arginine which bypasses the GIT (Flynn *et al.*, 2002).

In humans, decreased arginine in the diet does not affect growth or nitrogen balance in normal healthy infants; children or adults (Carey *et al.*, 1987; Castillo *et al.*, 1994), as *de novo* synthesis of arginine from citrulline is sufficient to maintain normal cellular metabolism (Carey *et al.*, 1987). However, inhibition of citrulline synthesis, the precursor of arginine, results in the arrest of normal growth in humans (Levillai n *et al.*, 1990). In contrast to healthy humans, arginine is indispensable for normal growth in young cats, dogs, chicks, ferrets and rabbits with urea synthesis defects (Carey *et al.*, 1987). In immature pigs, rats and guinea pigs an arginine deficient diet reduces growth and results in orotic aciduria (Carey *et al.*, 1987; Jeevanandam *et al.*, 1985; Alonso & Rubio; 1989). Adult rats showed elevated orotic acid (an intermediate in the biosynthesis of the pyrimidine nucleotides) urinary excretion when they were fed a diet lacking arginine (Visek; 1986). In addition, the ingestion of an arginine-free meal by strict carnivores rapidly leads to hyperammonaemia, encephalopathy and even death (Morris; 1985). Therefore, comparisons of amino acid dietary studies which extrapolate results from animals to humans must be regarded with caution.

1.4.7 Interventions to increase arginine concentration

Although, as discussed, arginine deficiency only occurs in stress situations such as the presence of ROS; the administration of arginine was found to reduce plasma homocystine concentrations (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982). Hence arginine was administered by intravenous infusion or orally in many cardiovascular studies in an attempt to alleviate hypertension. However, results were contradictory and often did not meet the expectations due to variations in the dosage and the time periods of administration. Moreover, the route of administration (infusion versus oral) is likely to have contributed to the inconsistent results.

1.4.7.1 Infusion of arginine in humans

Arginine is alkaline at physiological pH, hence in order to prevent an acid-base imbalance and to increase solubility, arginine hydrochloride (HCl) salt was used for intravenous infusions (Wu & Meininger; 2000). A rapid onset of hypotension was found when 30g (500mg kg^{-1}) of arginine was infused over 30 minutes in five healthy male volunteers. Their blood pressures returned to normal 20 minutes after arginine infusion was stopped. This hypotensive effect was due to NO release (Nakaki *et al.,* 1990; Cernadas *et al.,* 1992) from vascular endothelial cells. Infusion of arginine into healthy subjects and patients with primary hypertension caused a rapid decrease in systolic and diastolic blood pressure (Nakaki *et al.,* 1990; Ebel *et al.,* 1993; Moncada *et al.,* 1993) and prevented the development of hypertension in animals prone to the disease (Moncada *et al.,* 1993). Moreover, the administration of arginine was shown to reverse endothelial dysfunction associated with hypertension, diabetes mellitus, aging, smoking, hypercholesterolaemia and obesity (Wu & Meininger; 2000). In patients with severe CHF, the intravenous infusion of arginine enhanced their cardiac performance mainly by reducing their systemic vascular resistance (Bocchi *et al.,* 2000). Indeed, intravenous infusion of arginine was shown to increase vasodilation and reduce peripheral resistance in heart failure patients (Hirooka *et al.,* 1994, Kanaya *et al.,* 1999), and to increase NO production and decrease endothelial dysfunction in patients with coronary artery disease (Drexler *et al*; 1991; Tousoulis *et al*; 1997). Moreover, intra-coronary arginine infusion is reported to dilate atherosclerotic arteries in patients with coronary artery disease (Tousoulis *et al.,* 1998), and to restore endothelium-dependent vasodilation in the coronary microcirculation of hypercholesterolaemic patients (Drexler *et al.,* 1991). In patients with primary hypertension, an intravenous infusion of arginine is reported to improve systemic as well as renal hemodynamics (Maxwell & Cooke; 1998). In addition, irrespective of whether renal function was normal or insufficient, infusion of high doses of arginine [(30g) 500mg kg^{-1}] reduces blood pressure and renal vascular resistance (Higashi *et al.,* 1999).

The mechanisms of the beneficial cardiovascular effects of high dose arginine infusion are complex. The infusion of 500mg kg^{-1} arginine over 30 minutes (Nakaki *et al.,* 1990; Hishikawa *et al.,* 1993; Creager *et al.,* 1992) resulted in a 100 - 700 fold increase in circulating arginine (from 41 - 28 240µmol L^{-1}) within 20 minutes (Hishikawa *et al.,* 1993). The

physiological effects of such an enormous rise in arginine include the release of insulin (Floyd *et al.,* 1966) which may cause vasodilation (Creager *et al.,* 1985). Following an intravenous infusion of 30g arginine, GFR was reported to increase from 118±10 to 134±11ml min^{-1} (Smoyer *et al.,* 1991). This increase in GFR itself may have a hypotensive effect. In addition, growth hormone, glucagon and prolactin released by arginine (Nakaki *et al.,* 1990; Calver *et al.,* 1991; Floyd *et al.,* 1966) may cause vasodilation (Creager *et al.,* 1985). Thus the conclusion that high dose arginine infusion induces hypotension due to the release of NO (Nakaki *et al.,* 1990; Hishikawa *et al.,* 1993; Creager *et al.,* 1992; Ebel *et al.,* 1993) should be regarded with caution.

Although arginine infusion decreases blood pressure in hypertensive patients, no such effect is seen in patients with malignant hypertension (Sato *et al.,* 2000). This observed effect may be due to the severe impairment of the arginine/NO pathway in patients with malignant hypertension (Tousoulis; 2002). In another study intra-arterial arginine infusion did not improve endothelial function (Panza, Casino, Badar *et al.,* 1993), which provides evidence that endothelial dysfunction in hypertensive patients might not be associated with insufficient substrate availability for NO production (Taddei *et al.,* 1996). The reason for the controversial findings could be the different pathophysiological mechanisms of hypertension with the most important mechanism being the decrease of renal vascular resistance beyond its peripheral vasodilatory effects (Tousoulis; 2002).

1.4.7.2 Oral administration of arginine

Although oral administration of supplemental arginine is less effective than intravenously administered arginine (as only 50% of dietary arginine enters the systemic circulation), beneficial cardiovascular effects have been observed. Oral administration of arginine at a dose of 6.6g per day reversed endothelial dysfunction in hypercholesterolaemic patients (Maxwell, Anderson *et al.,* 2000), and six grams arginine orally per day for ten days in healthy adults partly abolished post-prandial endothelial impairment (Marchesi *et al.,* 2001). In addition, oral administration of arginine in patients with chronic heart failure at a dose of eight grams per day for four weeks improved endothelium dependent vasodilation (Hambrecht *et al.,*

2000). However, when arginine doses of 20g per day for four weeks were administered orally in heart failure patients, no improvement in endothelial cell function was observed (Chin-Dusting *et al.,* 1996). The lack of effect on endothelial function was attributed to decreased water solubility at the high dose (20g) used. The decreased water solubility may decrease the bioavailability of arginine compared to lower doses (Tousoulis; 2002). Nevertheless, oral administration of arginine at five to seven grams three times per day over a period of 12 weeks was reported to increase plasma arginine concentrations in healthy and in hypercholesterolaemic adults (Tangphao *et al.,* 1999).

1.4.7.3 Safety of arginine supplementation in humans

Administration of arginine in general is safe as it is not toxic. Intravenous arginine hydrochloride at doses up to 500mg kg^{-1} body weight for infants over 30 - 60 minutes had no side effects (McCaffrey *et al.,* 1995). Oral arginine-hydrochloride doses of 9g kg^{-1} body weight per day and intravenous infusions of up to 30g kg^{-1} body weight for 60 minutes had no serious side effects in adults (Creager *et al.,* 1992; Beaumier *et al.,* 1995; Miyazaki *et al.,* 1999). However, the most beneficial oral dose in cardiovascular disease was found to be 7g kg^{-1} body weight three times per day which only caused gastrointestinal complaints as side effects, due to increased NO production in the intestines (Goumas *et al.,* 2001), and impaired intestinal absorpsion of the cationic amino acids such as lysine and histidine. The solution to this gastrointestinal discomfort could be the alternative use of citrulline, which is the precursor for arginine synthesis (Wu, Meininger & Knabe *et al.,* 2000). Moreover, as excessive NO production leads to destruction of cells and could mediate the pathogenesis of autoimmune diseases, allograft rejections and septic shock (Cook & Cattell; 1996), it is not advisable to administer arginine to patients with severe infections, inflammatory- or autoimmune disorders, malignancy or pathological angiogenesis (Flynn *et al.,* 2002).

1.4.7.4 Administration of citrulline

The biological half-life of arginine in mammals is relatively short due to high arginase activity which rapidly degrades arginine in cells and tissues. Intravenous infusions of citrulline, which

can be used for arginine synthesis (Wu & Morris; 1998), were found to be more effective in increasing circulating arginine concentrations in pregnant ewes. Citrulline administration achieved a sustained prolonged increase in circulating arginine concentration in ewes (Lassala *et al.,* 2009) and the half-life of citrulline was found to be twice that of arginine (Lassala *et al.,* 2009). The lower half-life of arginine was due to the much higher activity of arginase on arginine compared to argininosuccinate synthase and argininosuccinate lyase responsible for citrulline catabolism (Wu & Morris; 1998). A study on ten healthy individuals who received an oral citrulline dose of 0.18g kg^{-1} per day, showed that oral citrulline can be used to increase systemic citrulline and arginine availability since citrulline is efficiently absorbed in the gut mucosa and reabsorbed in the kidney, hence little citrulline is lost in urine (Rougé *et al.,* 2007). Furthermore, the degradation by arginase of arginine derived from citrulline was found to be insignificant (Lassala *et al.,* 2009). These findings provide evidence for the complex metabolism of arginine via its compartmentalized pathways in mammals (Wu & Morris; 1998). In this regard, oral citrulline supplementation may be more beneficial in hypertension than arginine supplementation, but the effects of citrulline on the nitrogen balance of the body are not yet known (Rougé *et al.,* 2007). However, whether arginine or citrulline is administered intravenously or orally, the beneficial effects on endothelial function depend upon the efficient transport of these amino acids into endothelial cells. Hence, amino acid, particularly arginine transporters and the effects of alterations in these transporters on NO production warrant discussion.

1.4.8 Transport of cellular amino acids

In patients with CHF the clearance of L-[^{3}H]arginine after a 40 minute forearm intra-arterial infusion of 100nCi min^{-1} L-[^{3}H]arginine was significantly decreased compared with control individuals (64 ± 2 vs 133 ± 14ml min^{-1} $p = 0.002$). Similarly, in peripheral blood mononuclear cells (PBMC) isolated from patients with CHF, L-[^{3}H]arginine uptake was decreased compared to in PBMC's isolated from healthy control subjects. Indeed, in patients with CHF mRNA expression for the CAT-1 cationic amino acid transporter was reduced by 76%. These *in vivo* and *in vitro* findings of decreased arginine transport in CHF explain the beneficial effects of supplemental arginine on vascular function in CHF (Kaye *et al.,* 2000).

The regulation of arginine transport into endothelial cells is important in the biosynthesis of NO (Avila-Chavez *et al*., 1997) and hence plays an important role in the development of diseases such as hypertension, CHF, atherosclerosis, preeclampsia and renal failure.

Cellular uptake of a variety of amino acids in various tissues suggests that several different transporter systems exist with substrate specificities (Christensen; 1990). Initially it was difficult to assign specific amino acids to a particular transport system, but with newly developed molecular biology it became possible to identify the specific transporter proteins responsible for cellular uptake of specific amino acids (Avila-Chavez *et al*., 1997). Yet, transport of amino acids across cell membranes is a complex tissue- and species specific process involving gene expression of discrete and complex membrane-bound transporter proteins, with a variety of mechanisms of action, requirements and functions, hence resulting in speculation and controversy regarding their functional role.

In the kidney, arginine, lysine, cystine and ornithine share a common transporter (Dent & Rose; 1951; Foreman *et al.*, 1980; Bovee & Segal; 1984). In addition, homocysteine; homocystine and homocysteine-cystein disulphide use the same transport system as arginine and lysine (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982). Consequently, renal reuptake of lysine, arginine and cystine is decreased by homocystine (Foreman *et al.*, 1982), and renal excretion of homocystine is increased by the dibasic cationic amino acids arginine and lysine (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982). Hence, administration of dibasic cationic amino acids such as arginine may reduce circulating plasma homocystine concentrations (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982), thereby decreasing endothelial dysfunction and subsequent development of hypertension. Moreover, transport of arginine by cationic amino acid cell membrane transporters into endothelial cells is important in the regulation of endothelial function and vasodilation. In this regard, factors that influence the transport of arginine warrant discussion. Hence, I will describe the cationic amino acid transporters and their potential role in hypertension.

1.5 Role of cationic amino acid transporters in hypertension

1.5.1 Introduction

The transport of different classes of amino acids (ie: neutral, cationic, sulphonic and anionic) across cell membranes of mammalian cells is mediated by different transport systems (Christensen & Antonioli; 1969, White, Gazzola *et al.*, 1982; Van Winkle *et al.*, 1985; 1988; Devés *et al.*, 1992; Devés & Boyd; 1998; Closs *et al.*, 2004; Hatzoglou *et al.*, 2004) which have distinctive substrate specificities (Christensen; 1990). Although it was previously difficult to accurately assign specific amino acids to a particular transport system, with the more recent development of molecular biology the identification of specific transporters for particular amino acids has become possible (Avila-Chaves *et al.*, 1997). In this regard, cationic amino acids such as arginine, lysine and ornithine were previously thought to share the same transporter, termed the cationic amino acid transporter (White, Gazzola *et al.*, 1982; White; 1985). However, more recently five different cationic amino acid transporters have been recognised namely y^+, $b^{o,+}$, y^+L, $B^{o,+}$ (Avila-Chavez *et al.*, 1997; Devés & Boyd; 1998) and b^+ (Nawrath *et al.*, 2000; Mann *et al.*, 2003); where the exponents 'o' and '+' indicate neutral and positive charges respectively (Closs *et al.*, 2004). In endothelial cells the regulation of the transport of the cationic amino acids arginine and homocystine across the cell membrane is important in the biosynthesis of NO. As the main cationic transporters expressed in endothelial cells are the y^+ and the y^+L, for the purpose of this thesis I have concerned myself with these two cationic amino acid transporters.

1.5.2 The y^+ and y^+L transporters

Although there has been some confusion in the terminology of these transporters in the past, the y^+ transporter is now recognised as a low affinity system (high K_m) and the y^+L transporter as a high affinity system (low K_m). Hence, as shown in renal cortical tubule cells, at low amino acid concentrations the high affinity system becomes increasingly important; whereas at increasing concentrations of amino acids the low affinity system is the primary (major) transporter (Foreman *et al.*, 1980; 1982). In addition to differences in substrate affinity, these

two transporters differ with respect to the inhibition of substrate transport by competing amino acids. For example as shown in the renal cortical tubule cell, the high affinity system for cystine uptake was inhibited by amino acids such as lysine, ornithine, leucine and isoleucine, whereas the low affinity system was unaffected by these amino acids (Foreman *et al.*, 1980). In addition, cell membrane amino acid transport via the y^+ and the y^+L systems is both complex and specific as, different tissues and cell locations have been shown to express different transporters. For example, in cultured porcine pulmonary aortic endothelial cells both the y^+ and the y^+L systems have been shown to mediate arginine transport, whereas in the cell membrane vesicles of these cells, evidence of only the low affinity y^+ transport has been shown (Zharikov & Block; 1998). In contrast to rat hepatoma cell lines and human fibroblasts, the y^+ transporter contributes little to the cationic amino acid flux in rat hepatocytes (White, Gazzola *et al.*, 1982; White & Christensen; 1982a). However, the identification and characterisation of the y^+ and the y^+L membrane transporters is dependent upon the concentrations of the particular substrate and its competing amino acids. In this regard the lack of evidence of a high affinity transporter may be explained by the use of high concentrations of substrate, which are often higher than normal physiological concentrations (Devés *et al.*, 1992). It is not surprising that at high substrate concentrations only the low affinity y^+ transporter was identified. Indeed, evidence for the presence of the y^+LAT-2 (y^+L) transporter in brain cells has subsequently been shown in studies using low substrate concentrations (< normal physiological range) (Bröer *et al.*, 2000). In addition, there is a paucity of data on the impact of a range of concentrations of competing amino acids on the transport of a substrate over a range of substrate concentrations. Moreover, despite suggestions that transporter expression is tissue specific (Zharikov & Block; 1998; White, Gazzola *et al.*, 1982; White & Christensen; 1982b), the complexity of arginine transport by the endothelial cells (the primary site of NO biosynthesis) has long been overlooked.

It has been suggested that eNOS activity and thus NO production is dependent on the extracellular availability of arginine and on the uptake of arginine by the y^+ transport system (Sobrevia & Mann; 1997; Closs; 2002; Mann *et al.*, 2003). Indeed, the addition of arginine to the culture medium of PAEC increased the production of NO (Palmer *et al.*, 1988). Hence, it

is important to discuss what is known regarding the transport of the cationic amino acid, arginine.

In addition to the y^+ transporter of arginine, the y^+L transporter has a higher affinity for arginine (Forray *et al.*, 1995). Both the y^+ and the y^+L transporters are stereospecific for the L-isomers of amino acids, including arginine (Devés *et al.*, 1993; Forray *et al.*, 1995; White; 1985). Incubating rat cardiomyocytes with a NO specific fluorescent dye, diamino fluorescein (DAF)-diacetate (DA) at a concentration of 10μM for at least an hour, together with 15mM (15 000μM) L-arginine or D-arginine showed a sustained increase in fluorescence intensity by confocal fluorescence spectroscopy for L-arginine but not for D-arginine. Cellular uptake of arginine is thus stereospecific, as was confirmed by D-arginine failing to produce significant voltage currents compared to the voltage currents produced by the same concentrations of L-arginine in cardiomyocytes (Peluffo; 2007).

Although arginine uptake by endothelial cells is mediated by the y^+ and the y^+L transporters (Kakoki *et al.*, 2006), it was estimated that approximately 80% of arginine transport in endothelial cells is via the y^+ transporter (Patel *et al.*, 1996) and that the y^+L system plays a smaller role in arginine transport in endothelial cells due to the possibility of being inhibited by the presence of neutral amino acids such as leucine (Mann *et al.*, 2003). Therefore, the y^+L system was previously regarded as unimportant in the vasculature (Mann *et al.*, 2003). Furthermore, a genetic defect of the y^+ transporter has been linked to decreased arginine uptake and thus to endothelial dysfunction. Indeed, normotensive children of hypertensive parents have decreased arginine uptake and endothelial function, suggesting a genetic change in the function of the CAT-1 (y^+) transporter (Yang & Kaye; 2006). However, the importance of the high affinity y^+L system in the transport of arginine for the production of NO in endothelial cells when the y^+ system is inhibited should not be underestimated (Arancibia-Garavilla *et al.*, 2003). Indeed, in hypertension, transport by the y^+L system is reported to be decreased (Mendes-Ribeiro & Brunini; 2004; Schlaich *et al.*, 2004) while the y^+ system is unaffected (Mendes-Ribeiro & Brunini; 2004). Therefore, normally functioning y^+ transport could be responsible for the beneficial effect of arginine supplementation in hypertensive patients (Rajapakse & Mattson; 2009). Nevertheless, the role of these two transporters across

a range of arginine concentrations, as well as a range of concentrations of competing and thus inhibiting amino acids, such as homocystine and leucine has not been investigated. Therefore, the role of both the y^+ and the y^+L transporters in the transport of arginine, homocystine and leucine and the effects of these amino acids on the production of NO were investigated in this thesis.

1.5.3 Genes coding for y^+ and y^+L transporters

Two gene families, SLC7A and SLC3 encode cationic amino acid transport proteins resulting in six different gene products. The SLC7A genes code for the cationic amino acid transporters (CAT): CAT-1; CAT-2A; CAT-2B; CAT-3 which are cationic amino acid selective and exhibit the characteristics of the y^+ transporter system. The SLC3 genes code for the previously termed, BAT, and the 4F2hc broad scope (both cationic and neutral amino acids) transporters which are characteristic of $b^{o,+}$ and y^+L transport systems respectively (Devés & Boyd; 1998). In constitutive NO producing cells such as endothelial cells the y^+ system is the principal cationic amino acid transporter (Bogle *et al*; 1992, Durante *et al*; 1996). Nevertheless, transporters characteristic of the y^+L system are also expressed by endothelial cells. Indeed, in endothelial cells two cationic transporter systems have been described. The y^+ system components are due to CAT-1 and CAT-2B transporters, and the y^+L system components are due to 4F2hc/y^+LAT-1 and 4F2hc/y^+LAT-2 transporters (Sala *et al.*, 2002).

1.5.4 Characterisation of y^+ and y^+L transporters

In addition to differences in substrate affinity and specificity the y^+ and y^+L transporters have been characterised by kinetic studies (Devés *et al.*, 1992; Closs, Gräf *et al.*, 1997; Speake *et al.*, 2003; Arancibia-Garavilla *et al.*, 2003; Leoncini *et al.*, 2003; Signorello *et al.*, 2003; Chin-Dusting *et al.*, 2007); by sodium (Na^+) dependency or independency (White, Gazzola *et al.*, 1982; White; 1985; Greene *et al.*, 1993; Patel *et al.*, 1996; Durante *et al.*, 1996; McDonald, Rouhani *et al.*, 1997; Zharikov & Block; 1998; Estéves *et al.*, 1998; Kanai, Fukasawa *et al.*, 2000); by transport inhibitors such as *N*-ethylmaleimide (NEM) and 2-amino-2-norbornane-carboxylic acid, also known as β-2-aminobicyclo(2,2,1)heptanes-2-carboxylic acid (BCH)

(Foreman *et al.,* 1980; 1982; White, Gazzola *et al.,* 1982; White & Christensen; 1982a; White; 1985; Ewadh *et al.,* 1990; Greene *et al.,* 1993; Bussolati *et al.,* 1993; Patel *et al.,* 1996; Zharikov & Block; 1998; Mendes-Ribeiro *et al.,* 1997; 1999; Gräf *et al.,* 2001; Sala *et al.,* 2002; Casanello & Sobrevia; 2002; Arancibia-Garavilla *et al.,* 2003; Leoncini *et al.,* 2003; Signorello *et al.,* 2003; Martín *et al.,* 2006; Peluffo; 2007); or by amino acid inhibition and competition studies (Devés *et al.,* 1992; Bussolati *et al.,* 1993; Zharikov & Block; 1998; Avila-Chavez *et al*., 1997; Mendes-Ribeiro *et al.,* 1999; Kanai, Fukasawa *et al.,* 2000; Sala *et al.,* 2002; Nicholson *et al.,* 2002; Casanello & Sobrevia; 2002; Arancibia-Garavilla *et al.,* 2003; Leoncini *et al.,* 2003; Signorello *et al.,* 2003; Martín *et al.,* 2006). I will therefore discuss the characterisation of the y^+ and the y^+L transporters from studies which have used these different methodologies.

1.5.4.1 Transporter characterisation by sodium dependency

In order to determine the Na^+ dependency or independency of a transporter to transport cationic amino acids, choline chloride instead of sodium chloride is used in experimental solutions such as phosphate buffered saline (PBS) and Krebs Henseleit buffer (White; Gazzola 1982; White; 1985; Greene *et al.,* 1993; Patel *et al.,* 1996; Durante *et al.,* 1996; McDonald, Rouhani *et al.,* 1997; Zharikov & Block; 1998; Estéves *et al.,* 1998; Kanai, Fukasawa *et al.,* 2000). Depending on the Na^+ dependency or independency in the transport of cationic amino acids, the five cationic amino acid transporters were assigned to one of two classes as follows: the Na^+ independent systems y^+, y^+L, $b^{0,+}$, and b^+, in which cationic amino acids are taken up by carrier-mediated passive-facilitated diffusion, and the Na^+ dependent system $B^{0,+}$, coupled to the electrochemical plasma membrane gradient generated by Na^+ - K^+ ATPase (sodium-potassium ATPase) (Mann *et al*; 2003). Moreover the y^+ system is Na^+ independent and selective for cationic amino acid transport; whereas the y^+L system transports cationic amino acids independent of Na^+, but neutral amino acids dependent on Na^+ (Devés & Boyd; 1998; Devés, Angelo *et al.,* 1998). In particular the y^+LAT-1 transporter requires a positive charge for substrate binding (Kanai, Fukasawa *et al.,* 2000). The Na^+ dependence is beneficial for the y^+LAT-1 transporter in serving as an efflux path for cationic amino acids (eg. arginine), since low intracellular Na^+ concentrations cause cells to preferentially transport cationic amino acids

and not accept neutral amino acids. This is a mechanism for transporting cationic amino acids against an electrical gradient through plasma membranes (Kanai, Fukasawa *et al.*, 2000).

Although the transport of neutral amino acids by the y^+L system is Na^+ dependent, other cations have also been shown to play a role. For example Li^+ (lithium) proved to be an equally good cation substitute for Na^+, while K^+ was shown to be less effective than Li^+ (Devés *et al.*, 1993). Interestingly, the broad scope y^+L transporter changes into a cationic amino acid specific transporter in the presence of K^+. Indeed, in the presence of K^+, the y^+L transporter binds neutral amino acids with lower affinity and is less able to distinguish between neutral and cationic amino acids. In comparison, the y^+L transport of cationic amino acids is Na^+ independent and the rate of transport is not affected significantly when Na^+ is exchanged for Li^+ or K^+. The mechanisms underlying the differential effects of the cations on the y^+L transport of cationic *vs* neutral amino acids have been described by Kanai *et al.*, (Kanai, Fukasawa *et al.*, 2000).

There appears to be a structural relationship between the y^+ CAT's and 4F2hc/y^+LAT's. The y^+LAT-1 isoform is 30% structurally related at an amino acid level, to the y^+ cationic amino acid transporters CAT-1, CAT-2, CAT-3 and CAT-4 (MacLeod *et al.*, 1994; Devés & Boyd; 1998). Although CAT specifically binds cationic amino acid substrates, CAT-1 accepts neutral amino acids with low affinity in the presence of Na^+, indicating similarity to the y^+L transporter. Therefore, it is suggested that the CAT-1 and the y^+LAT binding sites recognise the positive charges on side chains of amino acids by a similar mechanism, consistent with the similar structure of these two proteins (Kanai, Fukasawa *et al.*, 2000). The system y^+L Na^+ recognition site with broad cation selectivity could be a primitive form of the less specific Na^+ binding sites of Na^+ independent transporters. Therefore, system y^+L transporters might be an evolutionary link between Na^+ dependent and Na^+ independent transporters (Kanai, Fukasawa *et al.*, 2000).

The y^+LAT-1 transporter is mainly expressed in epithelial cells and in the kidney while the y^+LAT-2 transporter has a much wider distribution. The y^+LAT transporters also mediate the uptake of arginine, leucine and glutamine; but arginine uptake was shown to be inhibited by

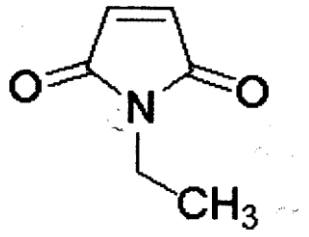
neutral amino acids in the presence of Na^+. Sodium-dependence is a typical characteristic of the y^+L system and of both y^+LAT transporters. However, only uptake of neutral amino acids was found to be Na^+ dependent. Arginine (a cation) uptake is not affected by the absence of Na^+. The uptake activities of the 4F2hc/y^+LAT-2 transporter [expressed in human copy DNA (cDNA) transfected *Xenopus laevis* oocytes] for arginine, glutamine and leucine were found to be similar, but the efflux activities for these three amino acids were different with the most rapid efflux seen for arginine in exchange for glutamine. Release of arginine by y^+LAT-2 is important in cells which produce NO by the NOS enzyme, since the extracellular arginine needed for NO production could be supplied by neighbouring cells releasing arginine in exchange for glutamine. This arginine/glutamine exchanger mechanism of y^+LAT plays an important physiological role in transferring arginine intercellularly and interorganally (Bröer *et al.,* 2000).

1.5.4.2 Transporter characterisation by the y^+ inhibitor *N*-ethylmaleimide

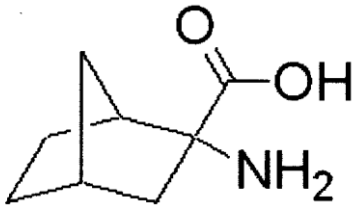
(Figure 1.3 upper panel).

N-Ethylmaleimide (NEM) has been used in many studies to inhibit the y^+ transporter in order to study amino acid transport by other cationic amino acid transporters not affected by NEM, such as the y^+L transporter (Foreman *et al.,* 1980; 1982; Patel *et al.,* 1996; Mendes-Ribeiro *et al.,* 1997; 1999; Zharikov & Block; 1998; Gräf *et al.,* 2001; Sala *et al.,* 2002; Aranciba-Garavilla *et al.,* 2003; Leoncini *et al.,* 2003; Signorello *et al.,* 2003; Martin *et al.,* 2006; Peluffo; 2007).

N-Ethylmaleimide is a sulfhydryl or thiol reagent (for structure see figure 1.3 upper panel) which reacts covalently with exposed sulfhydryl (-SH) groups (also called thiol groups) (Babu *et al.,* 2003). The precise location of the sulfhydryl groups and specifically their relationship to the active site of the transporter protein are important determinants of the susceptibility of the transporter protein to chemical sulfhydryl interactions (Patel *et al.,* 1996). The y^+ transporter has one sulfhydryl group whereas the y^+L transporter has two sulfhydryl groups (Estéves *et al.,* 1998) which is important in the understanding of the differences in amino acid



***N*-ETHYLMALEIMIDE**



2-AMINO-2-NORBORNANECARBOXYLIC ACID

Figure 1.3 Structure of *N*-Ethylmaleimide (NEM), inhibitor of the y^+ transporter (upper panel) and structure of 2-amino-2-norbornanecarboxylic acid (BCH), inhibitor of the y^+L transporter (lower panel).

transport by the y^+ and y^+L transporters. Indeed, in the y^+ transporter the presence of only one sulfhydryl group results in an exposed sulfhydryl group and hence the y^+ transporter is susceptible to inhibition by NEM. In comparison, in the y^+L transporter the two sulfhydryl groups are covalently bound by a disulphide bond hence rendering the y^+L transporter not susceptible to NEM inhibition. Various studies have shown the specificity of NEM for y^+ as opposed to y^+L transporter inhibition. These will be discussed below.

The transporter specificity of NEM was confirmed in a lysine (a cationic amino acid) uptake experiment, in which NEM inactivated the y^+ low affinity lysine transporter without having any effect on the y^+L high affinity transporter in human erythrocytes. Untreated erythrocytes revealed the presence of two transport systems in the lysine uptake experiments while the NEM treated cells revealed only the high affinity y^+L system, since the y^+ system is inhibited by NEM. Increased lysine substrate concentrations did not protect against this inactivation of the y^+ transporter by NEM (Devés *et al.*, 1993).

In HUVEC the co-incubation with NEM (inhibitor of the y^+ transporter) and leucine (a neutral amino acid which inhibits the y^+L transporter) resulted in decreased [^{3}H]-citrulline production (Arancibia-Garavilla *et al.*, 2003), which is an indicator of NO production (Moncada *et al.*, 1991). However, the incubation of HUVEC with NEM alone did not change [^{3}H]-citrulline production (Arancibia-Garavilla *et al.*, 2003), indicating that arginine (the precursor of citrulline) is taken up efficiently despite the inhibition of the y^+ transporter with NEM. Hence, arginine uptake in the presence of NEM must be via the y^+L transporter.

As the y^+ and y^+L transporters differ in their substrate affinity, the existence of a high affinity y^+L transporter of arginine was confirmed by assessing the impact of NEM on the transport of high versus low concentrations of arginine. NEM did not impact on the transport of 1.5μM arginine in HUVEC, but inhibited the transport of 100μM arginine. Since NEM is a system y^+ specific inhibitor these studies show that arginine transport is via the y^+L transporter when arginine concentrations are low but via the y^+ transporter when concentrations are high. Furthermore, arginine transport was saturable in the presence of NEM (y^+ specific inhibitor) and competitively inhibited by the neutral amino acid (y^+L inhibitor) leucine in the presence of

Na^+. Indeed, exposing HUVEC to NEM plus 1mM (1000μM) leucine, inhibited arginine transport completely. These results confirm that arginine transport is via both y^+ and y^+L transporters and support the suggestions that 0.1 - 10μM arginine could preferentially be transported by the high affinity y^+L system in endothelial cells and that leucine-sensitive arginine transport is due to the activity of y^+L transporters in HUVEC (Aranciba-Garavilla *et al.*, 2003).

1.5.4.3 Transporter characterisation by the y^+L inhibitor 2-amino-2-norbornane-carboxylic acid (Figure 1.3 lower panel).

2-Amino-2-norbornane-carboxylic acid (BCH), also known as β-2-aminobicyclo (2,2,1) heptanes-2-carboxylic acid (BCH) is a y^+L system analogue (for structure see figure 1.3 lower panel). Hence, BCH has been used in a number of amino acid transporter characterization studies to identify the role of the y^+L transporter in cellular amino acid uptake as BCH has been indicated to inhibit the y^+L transporter (Ewadh *et al.*, 1990; Greene *et al.*, 1993; Bussolati *et al.*, 1993) but has no effect on the y^+ transporter. However, results of BCH used as an inhibitor of system y^+L are discordant (see section 1.5.4.4 below), and should be read with caution.

1.5.4.4 Transporter characterisation by the y^+L inhibitor leucine

Although BCH, a selective substrate for system L has been regarded as an inhibitor of the y^+L transporter, in some studies BCH was found to have no significant effect (Devés *et al.*, 1993; White, Gazzola *et al.*, 1982). Importantly, BCH had an effect in HUVEC (Ewadh *et al.*, 1990; Bussolati *et al.*, 1993) and pulmonary endothelial cells (Greene *et al.*, 1993), but not in human fibroblasts or Ehrlich cells (White, Gazzola *et al.*, 1982), or human erythrocytes (Devés *et al.*, 1993). However, low concentrations of the neutral amino acid leucine inhibited the y^+L high affinity, NEM insensitive system (Devés *et al.*, 1993).

The neutral amino acid, leucine was noticed to inhibit the y^+L transporters at low concentrations, while BCH was found to have minimal effect (Devés *et al.*, 1993). Hence,

leucine is the preferred y^+L inhibitor used in transporter identification studies (Devés *et al.*, 1992; Zharikov & Block; 1998; Estéves *et al.*, 1998; Kanai, Fukasawa *et al.*, 2000; Nicholson *et al.*, 2002; Sala *et al.*, 2002; Casanello & Sobrevia; 2002; Aranciba-Garavilla *et al.*, 2003; Leoncini *et al.*, 2003; Signorello *et al.*, 2003; Martín *et al.*, 2006).

Inhibition of arginine transport by the neutral amino acid leucine is Na^+ dependent. The presence of leucine and Na^+ result in a higher K_m value for arginine but no significant change in V_{max}, which indicates competitive inhibition. Eadie-Hofstee analysis of leucine inhibition of arginine transport resulted in a linear relationship which is indicative of the role of a single transporter for arginine at a concentration range of 0.1 - 20μM in HUVEC. It is therefore possible that leucine-sensitive arginine transport is due to a high affinity transport system, in other words y^+L activity in HUVEC (Aranciba-Garavilla *et al.*, 2003).

1.5.5 Brief introduction to arginine transport and the effects thereof

The availability of arginine contributes to the function of the endothelial cell (Cooke *et al.*, 1991). Impaired arginine transport, leading to insufficient endothelial cell arginine concentrations, causes endothelial cell dysfunction and decreased NO production which leads to primary hypertension (Schlaich *et al.*, 2004). The low affinity y^+ transporter is the main transporter of arginine into endothelial cells (Patel *et al.*, 1996), while the high affinity y^+L transporter transports arginine too, but especially when the y^+ transporter is inhibited (Arancibia-Garavilla *et al.*, 2003) or when arginine concentrations are low (Devés *et al.*, 1992). Endothelial dysfunction is one of the pathogenic processes of cardiovascular diseases such as hypertension and heart failure. The mechanisms suggested to be involved in the pathology of these diseases may include impaired arginine transport and thus decreased NO production which leads to hypertension (Chin-Dusting *et al.*, 2007).

1.5.5.1 Role of high versus low affinity transporter systems in arginine transport

Although it was suggested in the 1980's that arginine is transported by two transporter systems, one active at extracellular arginine concentrations of 25 - 200μM, and the other

transporter system with K_m values in the millimolar range active at arginine concentrations above 1000μM (Christensen; 1985; White; 1985), the nomenclature and specific kinetics of these two transporters were not clarified. The kinetic analysis and identification of these two cationic amino acid transporters were first described by Devés *et al.*, in 1992 in human erythrocytes. The y^+ transporter was described as the low affinity cationic amino acid transporter and the y^+L transporter was identified as the high affinity transporter of cationic amino acids such as lysine at low concentrations (Devés *et al.*, 1992). Moreover, the K_m values for another cationic amino acid, arginine, were shown to differ three fold for the y^+ and the y^+L systems in human erythrocytes (Devés, Angelo *et al.*, 1998), with the y^+L transporter having the smallest K_m value and thus the highest affinity for arginine.

The presence in endothelial cells of the y^+ and the y^+L transporters originally identified in human erythrocytes, was confirmed by studies on the transport of arginine in the human umbilical vein endothelial cell line, SGHEC-7 (Mann *et al.*, 2003). Because the y^+ system is less sensitive to neutral amino acid inhibition than the y^+L system (Devés, Angelo *et al;* 1998; Palacín *et al.*, 1998), it was suggested that the influx of arginine is mainly through system y^+ in physiological conditions (Sala *et al.*, 2002).

1.5.5.2 Contribution of transporters y^+ and y^+L in cellular arginine uptake

Calculations based on the existence of two cationic amino acid transporters in rat cardiomyocytes indicated that at least half of the total transport of arginine is through the low affinity, high rate transporter at a cationic amino acid concentration of 0.6mM (600μM) and increases to 78% at a concentration of 3mM (3000μM) (Peluffo; 2007). It was estimated in endothelial cells, that the y^+ system transported approximately 80% of the total endothelial arginine (Patel *et al.*, 1996). Although the y^+L transporter system does transport cationic amino acids, the neutral amino acids such as leucine are preferred. Since arginine is the precursor of NO, the enhanced uptake or inhibition of arginine uptake by specific neutral amino acids and/or Na^+ sensitive transporters is important and hence may play a role in the development of hypertension and cardiovascular disease. Indeed, if arginine concentrations

are low it is more likely that the y^+L transporter would be playing a major role and the presence of neutral or competing amino acids may inhibit this transport.

1.5.5.3 Trans-stimulation and efflux of arginine (Figure 1.4).

The heavy chain BAT associates with the light chain $b^{o,+}$ to form the amino acid transporter, BAT/ $b^{o,+}$ which mediates system $b^{o,+}$ activity serving as an amino acid influx route. The 4F2 heavy chain (4F2hc) associates with the y^+LAT-1 or y^+LAT-2 light chains to form the 4F2hc/y^+L-like transporters. The 4F2hc/y^+LAT-1 and -y^+LAT-2 transporters serve as efflux routes for amino acids accumulated by the Na^+ dependent $B^{o,+}$ amino acid transporters in cells of the lung, kidney and intestines (Closs *et al.,* 2004). In renal proximal tubules, neutral amino acids enter the epithelial cells via the Na^+ dependent B^o system but leave the cell via the Na^+ independent system, whereas cationic amino acids leave the epithelial cells via the y^+L system of the basolateral membrane in the kidney tubules (Kanai, Segawa *et al.,* 2000). Therefore, it was noted to be most unlikely that 4F2hc/y^+LAT-1 would provide arginine to be used intracellularly as a substrate for NOS to produce NO (Closs *et al.,* 2004). This suggestion is supported by the finding that infused arginine normalized the decreased NO concentrations in a patient with lysinuric protein intolerance, which indicated that endothelial dysfunction was due to the low plasma arginine concentrations in the patient and not due to decreased arginine flux by the 4F2hc/y^+LAT-1 transporter in the endothelial cell membrane (Closs *et al.,* 2004). However, it was shown for the first time in HUVEC, that the high affinity y^+L system transport of arginine is necessary for NO synthesis in the human endothelium, but only when the y^+ system is inhibited by NEM (Arancibia-Garavilla *et al.,* 2003). Bearing in mind that at low arginine concentrations the role of the y^+ transporter system is minimal, the role of the y^+L transporter system is important. Hence, when plasma arginine concentrations are low, the role of the y^+ transporter needs to be investigated.

The transport of cationic amino acids by the y^+ system components has been described as bidirectional in endothelial cells (Sala *et al.,* 2002), while the y^+L system components y^+LAT-1 and y^+LAT-2 (Bröer *et al.*, 2000) are an efflux route for cationic amino acids in exchange for neutral amino acids (Verrey *et al.,* 2000), but also a major route for arginine uptake when

arginine concentrations are low (Speake *et al.,* 2003). Arginine was shown to have the highest affinity for the y^+LAT-2 intracellular binding site and therefore it was concluded that arginine efflux from cells might be the main function of the y^+LAT-2 transporter (Bröer *et al.,* 2000). In mammalian cells the transport of amino acids across the cell membrane is via means of transporter proteins. For transport of amino acids to occur, conformational changes in the transporter proteins are required. The conformational changes result in the translocation of the substrate binding site from one side of the cell membrane to the other (Krupka; 1985). The cis side of the cell membrane is the side of the higher substrate concentration and the trans side is the side of the lower substrate concentration (Dubyak; 2004). For amino acids these are the extracellular versus intracellular sides of the cell membrane respectively. Hence during amino acid uptake, the amino acid binding sites translocate from the cis to the trans side of the cell membrane (Closs, Albritton *et al.,* 1993).

The stimulation of cationic amino acid flux in the presence of cationic amino acid on the opposite side of the plasma membrane of the cell, is called trans-stimulation (Rajapakse & Mattson; 2009). Trans-stimulation is defined as the "movement" of a substrate from the side of the cell membrane where the substrate concentration is high (cis side), to the other side of the cell membrane where the substrate concentration is low (trans-side) (Dubyak; 2004). In trans-stimulation, excess extracellular cationic amino acids (other than arginine) or neutral amino acids enhance arginine efflux from the cells (Kakoki *et al.,* 2006; Rajapakse & Mattson; 2009). Hence, the availability of intracellular arginine is decreased and the production of NO by endothelial cells is reduced (Kakoki *et al.,* 2006; Rajapakse & Mattson; 2009). In contrast, excess extracellular arginine stimulates the production of NO by increasing the influx of arginine into the cell (Kakoki *et al.,* 2006).

There are differences between the cationic amino acid (y^+ system) transporter isoforms in their trans-stimulation effects. Cationic amino acid transporter-1 shows a strong trans-stimulation effect which indicates that it actually works better in the exchange mode than as a uniporter.

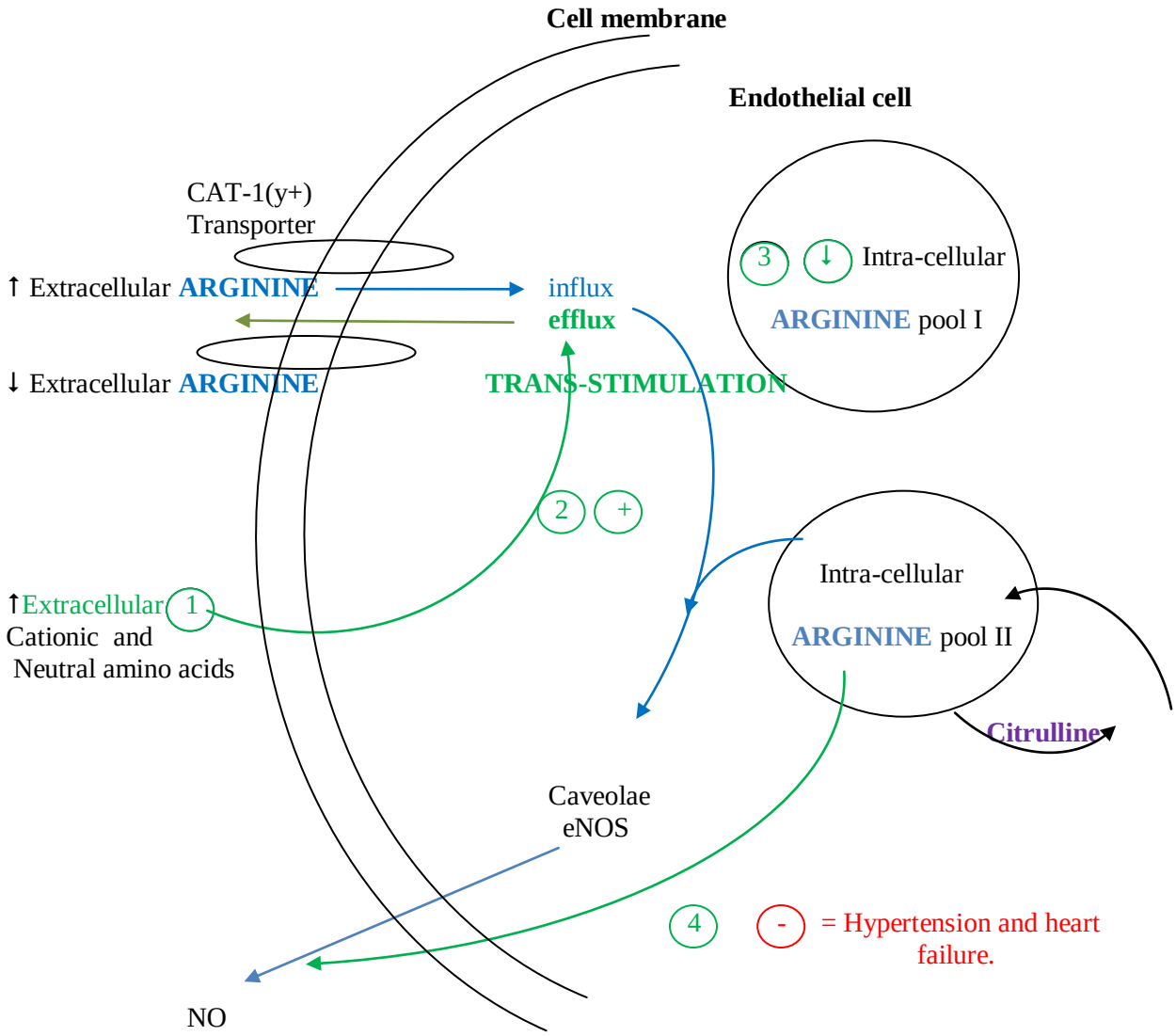


Figure 1.4 Arginine transport, intracellular arginine pools and nitric oxide (NO) production. Increased extracellular neutral amino acids (1) stimulate trans-stimulation and arginine efflux (2) resulting in decreased arginine in intracellular pool I (3) and hence arginine for nitric oxide (NO) production is accessed from intracellular pool II (4). Access to arginine from pool II is decreased in hypertension and heart failure.

Cells with the CAT-1 isoform will only be depleted of intracellular arginine when another cationic amino acid such as ornithine provided by arginase for example, is present extracellularly. This could be important in the regulation of arginine-dependent pathways (Closs *et al.,* 2004).

The CAT proteins (ie: y^+ transporters) transport arginine into and out of cells (Closs, Basha *et al.,* 1997). The arginine uptake by mouse CAT-1 and CAT-2, which includes the high affinity CAT-2A transporter, increased in the presence of arginine on the opposite (outside the cell) side of the cell membrane, which is in keeping with the behaviour of carrier proteins (Krupka; 1985). The trans-stimulation was faster when substrate is present on either side of the *Xenopus laevis* oocyte (transfected with the human copy DNA (cDNA) encoding CAT-2B transporter protein), membrane (Closs, Basha *et al.,* 1997), mouse macrophage and human endothelial cell (Closs, Scheld *et al.,* 2000). The return of the transporter to a conformation which allows efflux of arginine only occurs in the presence of high extracellular arginine (Closs, Albritton *et al.,* 1993). This could be a mechanism to protect cells from the depletion of arginine during starvation periods (Closs, Albritton *et al.,* 1993), as when extracellular arginine concentrations are minimal. Of interest, Closs showed that the mCAT-1 transporter was more dependent on trans-stimulation than the mCAT-2A transporter (Closs, Scheld *et al.,* 2000).

1.5.6 Nitric oxide and nitric oxide synthase regulation by y^+ and y^+L transporters

1.5.6.1 Role of y^+ versus y^+L transport of arginine for cellular nitric oxide production

Nitric oxide synthesis and transport of 1.5μM (ie. low concentration) arginine into HUVEC was inhibited by leucine in the presence of Na^+, suggesting that NO production depends on arginine uptake by the y^+L transporter system. These observations are supported by the finding that NEM (y^+ inhibitor) did not change NO production under the abovementioned conditions. These results are also in agreement with the suggestion that the increased y^+L activity found in platelets from chronic renal failure patients is associated with increased NO production (Mendes-Ribeiro *et al.,* 1999). Furthermore, eNOS inhibitors in HUVEC

significantly decreased NO production without affecting the transport of arginine by the y^+ system (Sala *et al.*, 2002; Flores *et al.*, 2003; Arancibia-Garavilla *et al.*, 2003), which suggests that the transport of arginine by the y^+ system does not limit NO production in these cells (Mann *et al.*, 2003). Moreover, the finding that the K_m for arginine transport by the y^+L system in HUVEC is 1.5μM which is close to the K_m values of eNOS at 3 - 10μM (Mann *et al.*, 2003), led to the hypothesis that altered y^+L activity rather than altered y^+ activity plays an important role in NO synthesis by eNOS (Arancibia-Garavilla *et al.*, 2003).

1.5.6.2 Membrane transporters, arginine pools and nitric oxide production (Figure 1.4).

As discussed in the section on arginine paradox, intracellular arginine is found in two pools (pool I and II). Pool I is readily exchangeable via CAT-1 and CAT-2A (y^+); whereas pool II is not. However, pool II is accessable for eNOS to produce NO when the arginine in pool I is decreased (Closs, Scheld *et al.*, 2000). The arginine in pool I may be decreased by increased extracellular amino acid concentrations; decreased extracellular arginine concentration or by decreased y^+ activity.

Nitric oxide production by vascular endothelial cells was shown to be dependent on the presence of exogenous arginine (Palmer *et al.*, 1988); even though endogenous arginine and hence NO can be produced intracellularly from the amino acid citrulline (Hecker, Mitchell, Harris *et al.*, 1990). Arginine transport into vascular endothelial cells is therefore important in NO production. Activation of the y^+ transport system was identified in HUVEC as a target for mediators which enhance NO production in endothelial cells (Bogle *et al.*, 1991; Bussolati *et al.*, 1993). Indeed, changes in the HUVEC y^+ transporter activity influence intracellular arginine concentrations and hence NO production (Bogle *et al.*, 1991; Bussolati *et al.*, 1993).

Interestingly, NO was shown to inhibit the uptake of its precursor arginine in PAEC, but the actual mechanism is not known (Patel *et al.*, 1996). Although the $B^{o,+}$ system usually transports neutral amino acids, this system can also transport arginine. Indeed, arginine Na^+-independent (system y^+) and Na^+-dependent (system $B^{o,+}$) transport decreased when cells were subjected to a premixed 95% N_2 plus 5% CO_2 gas containing 7.5parts per million NO for 12 -

24 hours. This NO inhibition of arginine uptake by endothelial cells was reversible within 24 hours. The Na^+ -dependent arginine transporter (system $B^{o,+}$) was found to be more susceptible to NO than to NEM inhibition (Patel *et al.,* 1996). In other words, NO limits its own production by inhibiting arginine transport into endothelial cells.

1.5.6.3 Arginine transport in vascular endothelial cells and vascular smooth muscle cells

Research showed that 70% of arginine uptake by BAEC and bovine aortic smooth muscle cells (BASMC) of the same vessel wall is Na^+ -independent, but that Na^+ does increase transport of arginine into both these cell types (Durante *et al.,* 1996). The uptake of arginine was biphasic in both cell types, indicative of the activity of both high (y^+L) and low (y^+) affinity transporters. The BAEC had a lower affinity for arginine but a higher capacity than BASMC. Both the low and high affinity transport systems in the BAEC showed a two to four fold higher K_m (indicative of a lower affinity) and a two fold higher V_{max} (indicative of a faster rate of uptake) for arginine than in BASMC (Durante *et al.,* 1996).

Endothelial NOS (eNOS) is incorporated in the endothelial cellular caveolae. Cationic amino acid transporter-1 (CAT-1) (y^+) preferentially delivers extracellular arginine substrate to eNOS located in these caveolae, for NO production in the presence of excess intracellular arginine. Processes which regulate CAT affinity could have a significant effect on NO generation by eNOS (Parnell *et al.,* 2004). However, as the uptake of arginine is biphasic, arginine transport activities of both systems y^+ and y^+L could be rate limiting for NO production in HUVEC. Therefore, both these transporters could be determining factors in pathological conditions such as diabetes mellitus, where the arginine-NO-pathway in endothelial cells is changed (Arancibia-Garavilla *et al.,* 2003).

Although some arginine analogues such as 2-amino-5,6-dihydro-6methyl-4H-1,3thiazine (AMT) specifically inhibit NOS without interfering with arginine transport, other arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME) competitively inhibit arginine transport (Closs, Basha *et al.,* 1997), limiting substrate availability via either y^+ or y^+L to the NOS enzyme.

1.5.6.4 **Arginine transport and nitric oxide production** (Figure 1.4).

Preincubation of cardiomyocytes with 1mM (1000μM) L-NAME which inhibits NOS, completely inhibited the effect of 15mM (15 000μM) arginine on fluorescence NO intensity. These data confirm the specific activity of NOS in producing increased NO fluorescence after addition of arginine and confirming the suggestion that arginine is converted to NO by NOS (Peluffo; 2007). Arginine transport regulation is essential as it supports many metabolic functions of which NO synthesis is one. Therefore, alterations in the regulation of the arginine transport system can result in impairment of NO production (Chin-Dusting *et al*; 2007). Impaired endothelial-dependent NO activity and arginine availability is likely to contribute to hypertension and atherosclerosis. Furthermore, restricted arginine availability leads to eNOS using oxygen as its principal substrate and hence produces superoxide and hydrogen peroxide (Tsai *et al.,* 1998). Increased superoxide concentrations and/or decreased NO concentrations and/or production of thiol-reactive free radicals (Patel *et al.,* 1996) result in endothelial dysfunction which is associated with a variety of vascular diseases such as hypertension and atherosclerosis (Mueller *et al.,* 2005; Briones & Touyz; 2010). Oxidative stress in part due to free radical production, many of which react with thiols, may affect arginine transport which could then result in reduced NO production and decrease subsequent NO-dependent biological functions (Patel *et al.,* 1996), especially when arginine concentrations are low. Importantly, thiol- or –SH groups inhibit the y^+ transporter which in turn would decrease arginine transport and hence NO production.

1.5.6.5 **Homocysteine transport and effects on arginine transport and hence nitric oxide production (**Figure 1.5).

The transporter system y^+L mainly transport neutral amino acids (Mann *et al.,* 2003), such as homocysteine, cysteine, cystine and glutamine (Ewadh *et al.,* 1988; 1990). Bearing in mind the Na^+ dependence of neutral amino acid transport by y^+L, the uptake of arginine is decreased by approximately 50% in the absence of Na^+. Similarly arginine uptake is decreased when the y^+L transporter is inhibited by 30mM BCH, a possible system y^+L inhibitor. As neutral amino acids compete with cationic amino acids for transport via the y^+L transporter, high

homocysteine, or perhaps homocystine concentrations, are likely to compete with arginine for transport via the y^+L transporter.

High concentrations (0.5 - 2.5mM or 500 - 2500µM) of homocysteine analogous to those found in patients with hyperhomocysteinaemia (0.4mM or 400µM) (Jin *et al.,* 2007), have a time dependent effect on arginine uptake via the y^+ transporter. Bovine aortic endothelial cells exposed to 0.5 - 2.5mM (500 - 2500µM) homocysteine for six hours show an increase in arginine uptake (Jin *et al.,* 2007). The regulation of arginine transport involves cell membrane potential (Parnell *et al.,* 2004) with arginine as the charge-carrying cationic amino acid responsible for these voltage currents (Peluffo; 2007). Hyperpolarization (higher voltage) by agents such as bradykinin increase arginine uptake (Bogle *et al.,* 1991) while depolarization (lower voltage) may cause decreased arginine uptake (Zharikov *et al.,* 1997). Therefore, the increased arginine uptake may be attributed to the homocysteine induced membrane hyperpolarization (Jin *et al.,* 2007).

In support of this mechanism of enhanced arginine uptake, Jin *et al.,* (2007) showed that citrulline formation (an indicator of NO production) was increased by homocysteine without changing eNOS or CAT-1 protein expression in BAEC (Jin *et al.,* 2007). However, although a six hour exposure of BAEC to high homocysteine concentrations showed an increase in NO production, a 24 hour exposure to the same concentrations of homocysteine led to an increase in ROS production and a subsequent decrease in NO (Jin *et al.,* 2007). When BAEC are exposed to high concentrations of homocysteine for 24 hours, ROS generation is enhanced (Tsen *et al.,* 2003). The ROS mediate oxidation of the free sulfhydryl groups in the y^+ transporter (Patel *et al.,* 1996), hence inhibiting y^+ arginine transport. Furthermore, BAEC exposed to high homocysteine concentrations for 24 hours showed decreased CAT-1 (y^+) protein expression. Therefore, 24 hours exposure of BAEC to high concentrations of homocysteine reduces the availability of arginine for eNOS to produce NO (Jin *et al.,* 2007), analogous to endothelial dysfunction. Indeed, in the presence of high homocysteine concentrations, addition of an anti-oxidant (eg. ascorbic acid) improved the transport of

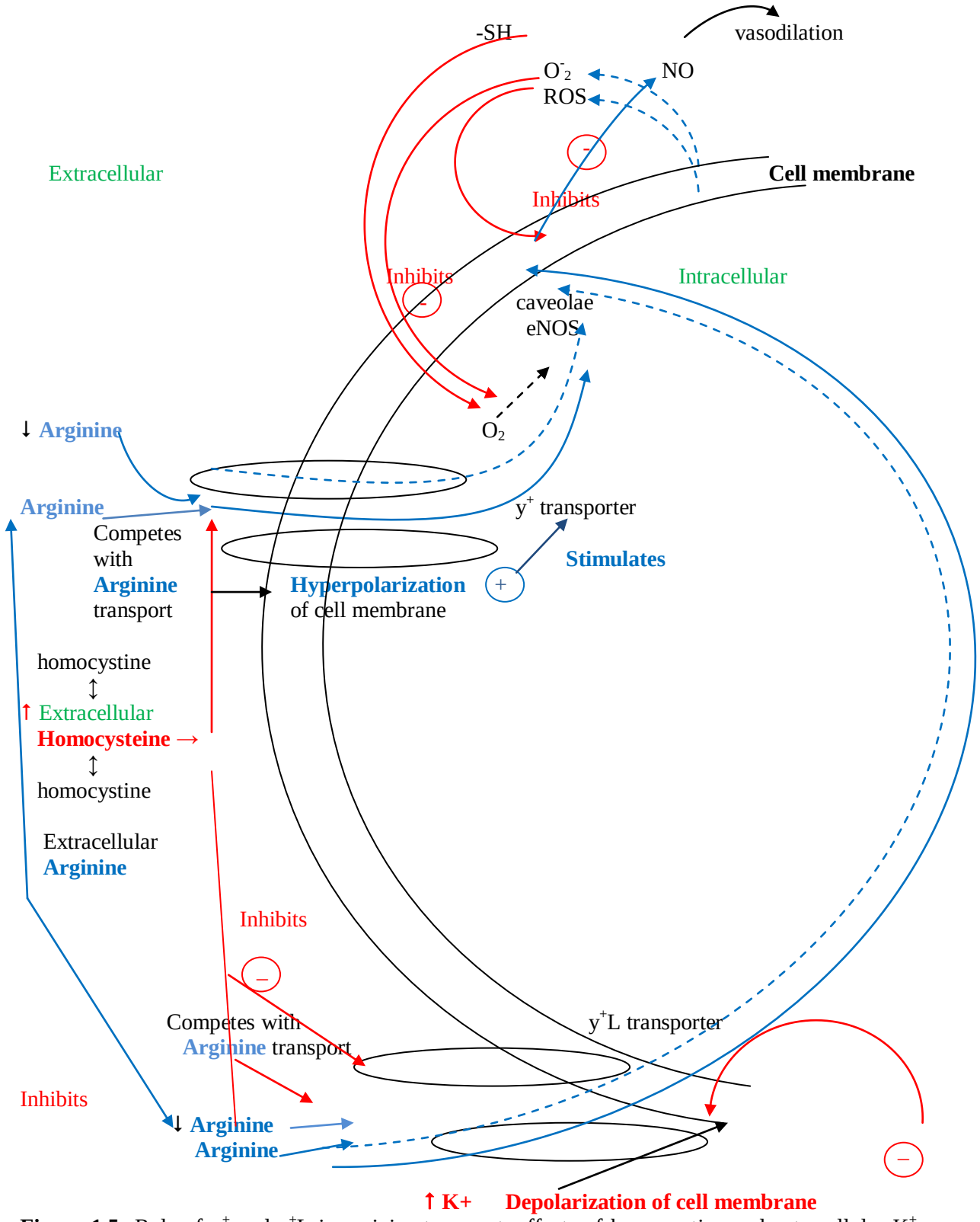


Figure 1.5 Role of y^+ and y^+L in arginine transport, effects of homocystine and extracellular K^+ on nitric oxide (NO) production by endothelial cells

arginine by the y^+ transporter in BAEC (Jin *et al.*, 2007). Moreover, limited availability of arginine causes eNOS to uncouple, hence increasing ROS production and exacerbating the adverse effects of high homocysteine concentrations on arginine transport. Hence, consistently high plasma homocysteine concentrations are implicated in endothelial dysfunction and cardiovascular disease.

In addition to the time dependent biphasic effect of high homocysteine concentrations on arginine transport, exogenous arginine concentrations influence homocysteine mediated ROS formation. Indeed, the increased peroxynitrite production by BAEC exposed to high homocysteine concentrations for 24 hours was prevented by exogenous arginine supplementation (Jin *et al.*, 2007). This data suggests that the inhibition of arginine uptake by high homocysteine concentrations not only depends upon the duration of exposure but also on the concentration of arginine. Nevertheless, data investigating the effects of high homocystine concentrations on arginine uptake and hence NO production at different concentrations of arginine are absent. It is possible that the effects of high homocystine and hence tHcy concentrations on endothelial function only manifest in the presence of low arginine.

1.5.7 Role of amino acid transporters in cardiovascular diseases

What evidence is there for a role of amino acid transporters in cardiovascular diseases? The presence of a defective transport of amino acids has been described in chronic renal failure (Mendes-Ribeiro *et al.*, 2001), preeclampsia (Dye *et al.*, 2001) and septic shock (Mendes-Ribeiro & Brunini; 2004). In addition, amino acid transporters have been shown to play a major role in congestive heart disease and hypertension (Kaye *et al.*, 2002; Yang & Kaye; 2006). In this regard, alterations in arginine transporters may limit the availability of arginine for NO synthesis. Indeed, endothelial dysfunction is commonly seen in patients with coronary artery disease, stroke or heart failure; and limited arginine availability for NO synthesis is thought to play a role (Chin-Dusting *et al.*, 2007).

A reduced rate of L-[^{3}H]arginine clearance from the circulation in the human forearm of patients with heart failure compared to control individuals is indicative of decreased

endothelial arginine transport. Moreover, the beneficial effect of arginine supplementation on the failing heart suggests that a deficiency of arginine may exist in heart failure. Decreased arginine transport by endothelial cells leads to intracellular arginine deficiency which in turn decreases NO production (Kaye *et al.,* 2000), leading to cardiovascular disease. A significantly lower V_{max} for arginine transport in patients with heart failure compared to control individuals was associated with a significant reduction in CAT-1 (y^+) mRNA expression (Kaye *et al.,* 2000). Indeed, there is a 38% decrease in mRNA expression of CAT-1 (y^+) in myocardium samples from CHF patients compared to healthy donor control samples (Kaye *et al.,* 2002).

In contrast, Mendes-Ribeiro described the capacity of arginine transport in erythrocytes by system y^+ to be 67% greater in CHF patients than in control individuals, while the y^+L capacity for arginine transport was unchanged (Mendes-Ribeiro & Brunini; 2004). However, the reduction in circulating arginine as described by Mendes-Ribeiro is in keeping with the observed beneficial effects of arginine supplementation on the vascular function in CHF patients (Hambrecht *et al*., 2000; Mendes-Ribeiro & Brunini; 2004).

Endothelial dysfunction due to decreased arginine transport (Schlaich *et al.*, 2004), which can be remedied with arginine supplementation, has also been associated with hypertension (Higashi *et al*., 1995). When hypertension was induced in healthy subjects by oral administration of 50mg hydrocortisone acetate, (which causes Na^+ retention) every 6 hours for 24 hours after five days on a diet containing 150mmol salt per day, no defects in arginine transport were found (Chin-Dusting *et al.,* 2003). This finding supports the hypothesis that arginine transport defects contribute to the pathology of hypertension and are not as a consequence of the development of hypertension (Chin-Dusting *et al.,* 2007). In addition, normotensive offspring of hypertensive individuals were found to have significantly reduced endothelial function and arginine transport, confirming the role of genetic changes in the function of the CAT-1 (y^+) protein (Yang & Kaye; 2006). Furthermore, arginine supplementation in normotensive offspring of hypertensive individuals is reported to improve endothelial function (Schlaich *et al.,* 2004).

In contrast, there is evidence that arginine transport into erythrocytes via the y^+L system is decreased in hypertensive patients and is associated with increased plasma arginine concentrations, while the y^+ system is not affected. Although system y^+L is a low capacity (rate) system, it accumulates intracellular arginine more efficiently over a long period of time than system y^+. Hence, down-regulation or impairment of y^+L activity could be the mechanism by which arginine available for NO production is reduced in hypertension (Mendes-Ribeiro & Brunini; 2004). As NO mediates the regulation of renal fluid volume, cardiac function and vascular tone, any change in the arginine-NO-pathway could cause hypertension (Rudd *et al.,* 2000 as cited by Mendes-Ribeiro & Brunini; 2004).

1.5.8 Explanations of discrepant findings

The literature on arginine and homocysteine transport by the y^+ and the y^+L transporters in cells is confusing due to the use of only one or a limited range of concentrations or concentration ranges above physiological concentrations of substrate or inhibitor. The use of different experimental techniques, different incubation times and different cell types contribute to the confusion. These variations in amino acid transport studies make the understanding of the importance of the role of arginine transport in cardiovascular disease difficult.

Although the physiological range for arginine and homocysteine in healthy individuals is from 100 - 200µM and 10 - 15µM respectively, in some arginine and homocysteine uptake studies BAEC were incubated with arginine in a range of 5 - 10 000µM for 10 - 15 minutes (Durante *et al.,* 1996) or with 500 - 2500µM homocysteine for 6 - 24 hours in the presence of 10% FCS (Jin *et al.,* 2007). The time and the addition of FCS would allow the conversion of reduced homocysteine to the oxidized dimers, such as homocystine as well as protein bound forms (Sengupta *et al.,* 2001). Amino acid transporter studies were also done on porcine pulmonary endothelial cells using 50µM and 10mM (10 000µM) arginine (Patel *et al.,* 1996), 100µM arginine only (Zharikov & Block; 1998) and a range of 0.005 - 10mM (5µM - 10 0000µM) arginine (Greene *et al.,* 1993) for 30 seconds. The higher concentrations of arginine are more likely to assess the function of the y^+ and the lower concentrations the function of the y^+L

transporter. Human erythrocytes were used to identify the cationic amino acid transporters (both y+ and y+L) using a range of 1 - 250µM substrate (Devés *et al.,* 1992) and human platelets were incubated with 1µM arginine to study the y^+L transporter (Mendes-Ribeiro *et al.,* 2007). Rat cardioventricular myocytes incubated with a range of arginine concentrations from 1 - 20mM (1000 - 20 000µM) indicated that at least 50% of arginine is transported by the y^+ transporter and the residual 50% by the y^+L transporter (Peluffo; 2007). Amino acid transporter studies in HUVEC include a study in which only 100µM arginine was used for 30 seconds (Sala *et al.,* 2002) and studies in which a range (7.5 - 1000µM and 15 - 1000µM respectively) of arginine concentrations were used for 1 minute (Casanello & Sobrevia; 2002; Arancibia-Garavilla *et al.,* 2003). Results of these studies are not in agreement and therefore some possible explanations for the discrepant results were postulated in the literature.

In addition to the large discrepancies in the range of concentrations of homocysteine and arginine assessed, divergent results obtained in the same cell type (HUVEC) could be due to the use of secondary subcultures (Bussolati *et al.,* 1993) opposed to primary cultures as used by Ewadh *et al.,* 1990 and Mann *et al.,* 1989 (Bussolati *et al.,* 1993). Discrepancies in transporter findings on HUVEC could also be due to differences in experimental conditions such as cells being confluent opposed to subconfluent at the time of the experimental procedure, the use of 20% opposed to 40% serum in the culture medium and whether or not there was a change to serum free medium 24 hours before the uptake study (Arancibia-Garavilla *et al.,* 2003). Indeed, environmental conditions could lead to differences in transporter results in the same cell type, for example, amino acid transporter activity was found to be increased upon cellular amino acid starvation of HUVEC (Bussolati *et al.,* 1993), but in studies by Ewadh *et al.,* 1990 and Mann *et al.,* 1989, very low amino acid transporter activity was detected in HUVEC due to omission of an amino acid starvation period (Bussolati *et al.,* 1993). Importantly, differences in cell culture conditions and/or experimental conditions can influence cell membrane potential. Indeed, Mann *et al.,* (2003) confirmed in a review article on amino acid transporters that discrepancies in results could be explained by differences in cell culture conditions and/or experimental conditions and/or differences in membrane potential (Mann *et al.,* 2003). Therefore, it is possible that cell membrane potential determines the extent to which arginine is accumulated in a cell. In confirmation of Mann's

work (Mann *et al.,* 2003), the rate of arginine influx into HUVEC was found to be inversely dependent on the extracellular K^+ concentrations (Bussolati *et al.,* 1993), and hence the degree of polarization. The fact that arginine transport is sensitive to conditions which cause depolarization, suggests that the membrane potential determines the extent to which arginine accumulates in the cells. Changes in depolarizing charges of the membrane potential result in decreased activity of the cationic transporters and hence reduce the intracellular arginine concentrations which in turn can decrease the production of NO (Bussolati *et al.,* 1993).

To further substantiate the suggestion that discrepant arginine transport results could be due to different endothelial cell sources, it was demonstrated that different venous umbilical cord endothelial cell types expressed different cationic amino acid transporter genes. The EA.hy296 human umbilical vein endothelial cells, which were transformed by hybridization with a lung carcinoma cell line, only expressed CAT-1 (y^+ system) (Closs, Scheld *et al.,* 2000) whilst the primary human umbilical vein endothelial cells (HUVEC) expressed CAT-1 and CAT-2B (y^+ system) (Casanello & Sobrevia; 2002; Sala *et al.,* 2002) as well as y^+LAT-1 and y^+LAT-2 (y^+L system) (Sala *et al.,* 2002). Furthermore, arterial- and venous endothelial cells also differ in arginine transport as was shown in BAEC, which is an arterial endothelial cell source, mainly transporting arginine by the y^+ system while the y^+L system is not operative (Ogonowski *et al.,* 2000), in comparison to both the y^+ and y^+L systems being active in some venous endothelial cells such as HUVEC (Sala *et al.,* 2002).

In an attempt to clarify the discrepant amino acid transporter findings I used a human umbilical vein primary endothelial cell model, HUVEC as well as a continuous human umbilical vein endothelial cell line, ECV_{304} (naturally transformed human umbilical vein endothelial cells from donor number 304). Both of these cell types are reported to express both y^+ and y^+L transporters. In order to address the discordance in the literature of cationic amino acid transporters and their role in the transport of arginine and in NO production, I studied the initial radiolabelled arginine uptake across wide ranges of concentrations of arginine and homocystine. Specifically, I chose to study the initial rate (30 seconds) of arginine uptake since data showed that the initial rate of uptake was linear. Furthermore, I will investigate the impact of low versus high homocystine concentrations on arginine transport in

the presence of low versus high arginine concentrations. Therefore, for the purpose of this thesis, the initial uptake of arginine at concentrations below, at and above the physiological concentrations of arginine in the absence or presence of homocystine, by the y^+ and the y^+L transporters in HUVEC and ECV_{304} in relation to NO production were investigated. The consistency of the incubation time (30 seconds), the wide range of arginine (1 - 2000µM) and homocystine (1 - 200µM) concentrations and the use of the primary HUVEC and the cell line ECV_{304} from the same source (human umbilical vein) may assist in clarifying the results and conclusions on the role of the y^+ and y^+L transporters in the transport of arginine in hypertension.

1.6 Role of antihypertensive agents in endothelial dysfunction and in hypertension

For the treatment of hypertension, there are currently a number of classes of antihypertensive agents available, including beta-blockers; calcium channel blockers; diuretics; angiotensin converting enzyme inhibitors (ACEI); angiotensin II receptor blockers; aldosterone receptor antagonists; central acting agents and alpha blockers. Although, all of these agents have been shown to be efficacious in the lowering of blood pressure (Chobanian; 2008), they differ in their degree of efficacy (Materson *et al.*, 1993; Ramsay *et al.*, 1999; Chobanian; 2008). Moreover, one particular class of antihypertensive agents, namely the ACEI, has been shown to have effects beyond blood pressure lowering (Lonn; 2002). These blood pressure independent effects, termed pleiotropic effects are also observed with angiotensin II receptor blockers (Delles *et al.*, 2002). Most other classes of antihypertensive agents have not been shown to have cardiovascular effects which are independent of their blood pressure lowering effects (Schiffrin & Deng; 1995; Delles & Schmieder; 2004). However, the more recently developed third generation $beta_1$ selective blockers (eg. nebivolol) are reported to stimulate NO release (Tzemos *et al.*, 2001), which is thought to be mediated by a reduction in ADMA concentrations in hypertensive patients (Pasini *et al.*, 2008). The pleiotropic effects of the ACEI and angiotensin II receptor blockers are mainly on the vasculature (Rizzoni *et al.*, 1998; Schiffrin & Deng; 1995). Importantly, ACEI are responsible for improvements in endothelial function (Buikema *et al.*, 2000) and beneficial vascular remodelling (Galderisi & De Devitiis;

2008). Moreover, antihypertensive agents that increase the bioavailability of NO may reduce target organ damage in hypertension (Portaluppi *et al.*, 2004).

Having established that ACEI indeed do have effects beyond blood pressure lowering, a number of investigators explored the potential mechanisms of these effects. In this regard the beneficial effects of ACEI on the vasculature have been attributed to the stimulatory effects of ACEI on eNOS; increased bradykinin and hence NO production; and the ability of ACEI to scavenge ROS (oxygen free radicals). Indeed, ACEI have been shown to restore the functioning of eNOS when it has been impaired by increased oxidative stress (Galderisi & De Devitiis; 2008). As a consequence of the stimulatory effects of ACEI on eNOS, NO production is enhanced (Balligand *et al.*, 2009). The ability of ACEI to reduce oxidative stress has been attributed to the sulfhydryl groups, present in some ACEI's, as sulfhydryl groups are known to scavenge superoxide anions (Simic; 1988). Indeed, sulfhydryl containing ACEI have been shown to produce anti-oxidant effects (Napoli *et al.*, 2004). However, not all ACEI contain sulfhydryl groups. For example, the ACEI captopril contains a sulfhydryl group, but enalapril does not (Jacoby & Rader; 2003; Unger; 2002). Nevertheless, both of these ACEI have been shown to increase NO production to a similar extent. A study in HUVEC reported that captopril increased NO production by 65% and enalapril by 64% (Desideri *et al.*, 2008). These similar effects of captopril and enalapril on NO production would seem to argue against a major role of the sulfhydryl groups in mediating the beneficial effects of ACEI's on endothelial NO production. Importantly, all types of ACEI's (ie. lipophilic or non-lipophilic), irrespective of whether they contain sulfhydryl groups, have been shown to have beneficial effects on endothelial NO production (Desideri *et al.*, 2008; Scribner *et al.*, 2003). The mechanisms of beneficial effects of ACEI on NO production therefore warrant further investigation.

Bearing in mind that arginine is the substrate for vascular NO production (Kakoki *et al.*, 2004), and hence is an important determinant of NO production especially in the presence of endothelial dysfunction, such as occurs in hypertension (Panza *et al.*, 1995); the activity of arginine transporters may play a role. Indeed, studies have reported decreases in the activity of either y^+ (Yang & Kaye; 2006) or y^+L (Moss *et al.*, 2004) transporters in hypertension. In

this regard, ROS have been shown to mediate oxidation of the free sulfhydryl group in the y^+ transporter (Patel *et al.,* 1996), the effects of which would be a decreased arginine transport via this transporter. Hence, the anti-oxidant properties of at least the sulfhydryl containing ACEI may produce beneficial effects on the y^+ transporters. In apparent conflict with these findings, are studies showing that angiotensin II increases the expression and activity of y^+ transporters (Hultström *et al.,* 2009; Low & Grigor; 1995). As ACEI result in decreased angiotensin II production, it could be argued that non-sulfhydryl containing ACEI's (which therefore lack anti-oxidant properties) may reduce arginine transport. Hence, the question remains as to the mechanisms responsible for the increased NO production mediated via ACEI in the absence of anti-oxidant effects. Although in endothelial cells, arginine is transported by two transporters, y^+ and y^+L (Kakoki *et al.,* 2006), the effects of ACEI on the y^+L (high affinity) transporter have not previously been investigated. In addition, despite reports of decreased function of arginine transporters in hypertension (Yang & Kaye; 2006; Moss *et al.,* 2004; Schlaich *et al.,* 2004), the effects of antihypertensive agents on the arginine transporters (y^+ and y^+L) have not previously been investigated. As extracellular arginine concentrations are reported in some studies to be increased in hypertension (Moss *et al.,* 2004; Perticone *et al.,* 2005), possibly due to decreased transport (Chin-Dusting *et al.,* 2007); and/or other amino acids such as homocysteine (Jin *et al.,* 2007) and ADMA (Perticone *et al.,* 2005) which compete for the same transporters, may be increased, the potential effects of ACEI and other antihypertensive agents on arginine transporters warrant investigation. "Hence, the aim of this part of my thesis was to investigate the effects of a sulfhydryl versus a non-sulfhydryl containing ACEI, as well as the effects of some antihypertensive agents on arginine transport by the y^+ and y^+L transporters. For these studies I selected two agents from the ACEI class, (one containing a sulfhydryl group, captopril, and another without a sulfhydryl group, enalapril), and a diuretic, hydrochlorothiazide (HCTZ).

1.7 Objectives and aims of my studies

1.7.1 Summary of problem statements

As described in the previous sections of this chapter, there is uncertainty regarding a) the role of arginine and its endothelial transport in the production of NO; b) the effects of homocystine on arginine transport; and c) the impact of sulfhydryl and non-sulfhydryl ACEI class of antihypertensive agents on arginine transporters and hence NO production. Moreover, there is much controversy on kinetics of the endothelial cell transporters of arginine and homocystine. Hence, in the present thesis I aimed to evaluate:

1) The endothelial cell transport of arginine using a careful kinetic analyses approach for analysing arginine transport, with and without cellular uptake modifiers, by a two transporter cell membrane system. (These data are described in chapter 3)
2) The impact of various concentrations of homocystine on the endothelial transport of arginine by the two transporters, and hence on the production of NO (These data are described in chapter 4)
3) The impact of two ACEI (sulfhydryl and non sulfhydryl) as well as hydrochlorothiazide (a common diuretic) antihypertensive agents on the two arginine transporters (These data are described in chapter 5).

CHAPTER 2

Basic materials and methods for cell culture, arginine uptake and nitric oxide production

For all studies included in my thesis, I used ECV_{304} and HUVEC cultures. In essence the methods followed to answer each of the objectives have the same basis and only differ moderately. Hence, in this chapter I will describe the basic materials and methods which are common to each of the subsequent chapters. In each of the individual chapters three, four and five I have therefore only included detail of the methods unique to that particular chapter.

2.1 Motivation for choice of endothelial cell type

2.1.1 ECV_{304} cells

Although human primary endothelial cell cultures would have been the ideal for these studies, the number of experiments that could have been performed would have been limited, as these cells have a short lifespan of only a few weeks, and their yield upon isolation from human umbilical cord vein is low. Moreover, the high cost of the growth factors required for culturing and proliferation of these cells would also have been limiting. Another disadvantage of donor obtained cells is the possibility of physiological and cellular differences between individual donors which could complicate or compromise consistency of experiments. Therefore, in order to limit cellular variation and to ensure experimental reproducibility in a variety of experiments over an extended period of time, I decided to use a single source, commercially available human endothelial cell line.

The spontaneously transformed immortal human umbilical cord vein endothelial cell line, ECV_{304}, was obtained from the American Tissue Culture Collection (ATCC) (ATCC, Rockville, Maryland, USA) and is described in the catalogue as follows. ATCC CRL-1998 ECV_{304} cells are spontaneously transformed immortal human umbilical cord vein endothelial cells isolated from donor number 304. These cells are characterised by a cobblestone monolayer growth pattern, a high proliferation rate without requiring any specific growth

factors and anchorage dependency (only grow when adhered to culture flask) with contact inhibition (stop growth on contact with other cells). Identifying these cells as endothelial in origin involved positive immunocyto-chemical staining for both PHM5 (glomerular epithelium monoclonal antibody and anti-human endothelium) and lectin Ulex europaeus-I (UEA-I), ultrastructural identification of Weibel-Palade bodies typical of endothelial cells and angiotensin-converting enzyme (ACE) activity was demonstrated. Cells were negative for factor VIII-related antigen.

Controversies about the use of ECV_{304} cells as a model for vascular endothelial cells exist. Although I obtained these cells from ATCC in 1998 as a spontaneously transformed immortalised human umbilical cord vein endothelial cell line, a subsequent study of the effect of homocysteine on the NOS pathway in ECV_{304} cells, referred to these cells as 'non-vascular' and stated that 'the ECV_{304} cell line is a derivative of the human bladder carcinoma line T24/83' (Stülinger *et al.*, 2001). Furthermore, Brown *et al.*, (2000) concluded that these cells are useful in receptor pharmacology studies due to their endothelial characteristics, but as they are genetically identical to T24/83 bladder carcinoma cells and not of HUVEC origin, they are inappropriate to study endothelial cell biology (Brown *et al.*, 2000). However, in a phenotypic characterization study of the ECV_{304} cells and the T24/83 cells, the ECV_{304} cells were found to express endothelial features, such as cobblestone morphology, anchorage dependency with contact inhibition and the presence of Weibel-Palade bodies typical of endothelial cells, and therefore, it was concluded that due to the scarcity of suitable endothelial cell line, ECV_{304} cells are an attractive *in vitro* model for endothelia (Suda *et al.*, 2001).

In personal communication with ATCC in 2007 I learned that the controversy about the ECV_{304} cells originated from the possibility that these cells could accidentally have been contaminated with a human bladder carcinoma cell line in the laboratory where the ECV_{304} were first cultured. Nevertheless, in my experience, I observed that ECV_{304} cells had many of the described endothelial characteristics such as cobblestone morphology, anchorage dependency with contact inhibition, NO production and positive flow cytometric labelling with the endothelial specific marker, CD62E (cluster designation 62 endothelial) conjugated to the PECy5 (phycoerytherin cyan 5) fluorescent dye.

In a review article, Mann *et al.,* (2003) mentioned the ongoing debate concerning the validity of the ECV_{304} cell line as endothelial cells. As ECV_{304} cells have an identical genotype to the T24/83 bladder carcinoma cell line, Mann *et al.,* (2003) argues against the use of ECV_{304} cells for endothelial studies. Nevertheless, since this review, scientists have continued to use ECV_{304} cells in eNOS/NO production studies and have referred to these cells as 'human umbilical vein endothelial cells' (Qiao *et al.,* 2006) or 'human vascular endothelial cells' (Huang *et al.,* 2007).

Recently a comparative study of the effects of protein kinase C on the y^+ and the y^+L transporters in different human cell types used quantitative mRNA PCR to determine the relative expression of these transporters in the various cell types including $ECV_{304,}$ HUVEC and EA.hy926 [human endothelial cells fused with human lung cancer cells (hybrid 926)] cells (Rotmann *et al.,* 2007). Of these three cell types the ECV_{304} cells expressed the most hCAT-1 (y^+ transporter) protein. In comparison, EA.hy926 expressed only a third of that expressed by ECV_{304}, and HUVEC expressed two thirds. HUVEC also expressed hCAT-2B protein but at a low level, and none of these three cell types expressed any hCAT-3 protein. The ECV_{304}, EA.hy296 and HUVEC cells almost uniformly expressed y^+LAT-2 (y^+L transporter) protein. Importantly, comparing arginine uptake at physiological concentrations (100µM) by these three cell types, the y^+ system contributed to 50-60% of the total arginine uptake by both ECV_{304} and HUVEC cells, but only to 33% in EA.hy926 cells (Rotmann *et al.,* 2007), and approximately 40-50% of arginine was transported by the y^+L transporter in both ECV_{304} and HUVEC cells. Moreover, the transport activity of the y^+ and the y^+L systems in ECV_{304} cells (59 and 41% respectively) did not differ significantly from that in the primary endothelial cells (HUVEC, 56 and 44% respectively), (Rotmann *et al.,* 2007). Hence, with respect to arginine uptake and the role of y^+ and y^+L transporters, no differences between ECV_{304} and HUVEC cells were observed. Based upon the similarity of ECV_{304} to HUVEC cells in the mechanisms of arginine uptake (Rotmann *et al.,* 2007), the ECV_{304} cell line is certainly valid as an endothelial cell line to assess arginine uptake and NO production. However, it is important to keep in mind that the % uptake of physiological concentrations (±100µM) arginine by the different transporters present in different cell types, could vary when using different types of culture media as well as when using later passages of the same cell line.

2.1.2 EA.hy926 cells

In the light of the abovementioned controversies on the ECV_{304} cell line, I obtained another human umbilical vein endothelial cell line, EA.hy926, kindly donated by Dr. Cora-Jean S Edgell (edgellcj@med.unc.edu, Pathology, University of North Carolina, Chapel Hill, NC, USA). The continuous human endothelial cell line, EA.hy926, was derived by fusing human umbilical vein endothelial cells with the continuous human lung cancer cell line designated A549. Hybrid cells produced a continuous human cell line expressing at least one highly differentiated vascular endothelial characteristic namely, Factor VIII-related antigen (Edgell *et al.*, 1983). In pilot studies of flow cytometric analysis on the effect of shear stress on NO production, by means of a NO sensitive dye, DAF-2/DA, I found that the EA.hy926 cells produced approximately 7% higher levels of NO than the ECV_{304} cells. Furthermore, flow cytometric analysis also indicated that NO production by both the EA.hy926 and ECV_{304} cells approximately doubled after shaking the cells at 37ºC for 24 hours, confirming that shear stress did increase NO production in both of these endothelial cell types. Unfortunately, the EA.hy926 cell line stopped proliferating during the course of the pilot study and hence was no longer viable for future experiments.

2.1.3 HUVEC

Bearing in mind the aforementioned criticisms of ECV_{304} cells and the failure to culture EA.hy926 cells for the NO production experiments, I purchased non-transformed, commercially available primary endothelial cells, called Human Umbilical Vein Endothelial Cells (HUVEC) (Lonza, Walkersville, MD, USA, via Whitehead Scientific, Brackenfell, SA). HUVEC are normal human umbilical vein endothelial cells derived from 12 pooled donor umbilical cords testing non-reactive by a FDA (Food and Drug Association) approved method for the presence of HIV-1, Hepatitis B and Hepatitis C viruses. These cells required the addition of growth supplements as described in section 2.2.3 below. These primary cultures were sustained successfully for eight passages, allowing for comparison of arginine uptake kinetics (see Chapters 3, 4 and 5) and NO production (see Chapter 4) in HUVEC *vs* ECV_{304}

cells in response to different arginine concentrations in the presence or absence of homocystine.

2.2 Cell culture methodology (Figure 2.1).

2.2.1 ECV_{304} culturing

I originally received a 1ml ampoule of cryogenically preserved ECV_{304} cells at passage number 129, at a concentration of 5 x 10^5 cells per 1ml of cryopreservation medium. On receipt the cells were thawed by manual swirling in a water bath at 40ºC. Once the cells were thawed, the cell suspension obtained was resuspended by carefully pipetting up and down three to four times with a 1ml disposable sterile graduated pipette (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA) to evenly disperse the cells. The whole 1ml cell suspension was added to 24ml complete medium 199 (M199) culture medium (Highveld Biologicals, Sandringham, Johannesburg, SA) in a 25cm^2 (125ml) cell culture flask (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA) and warmed to room temperature (± 20ºC). The M199 culture medium was supplemented with 10% heat inactivated foetal calf serum (FCS) (Gibco, The Scientific Group Adcock Ingram, Midrand, Johannesburg, SA) and a 50:50 mixture of 1% Penicillin and 1% Streptomycin (Pen/Strep) (Whitehead Scientific, Brackenfell, SA), and thus was called complete M199 culture medium. The Pen/Strep mixture was added to the M199 to prevent possible bacterial contamination. Prior to use the FCS was heat inactivated in a water bath at 56ºC for 45 minutes to destroy the naturally occurring serum complement which could perforate cell membranes and thus affect experimental observations.

The flask containing the cells was then placed in an incubator (Forma Scientific, Labotec, SA) set at 37ºC with 5% CO_2. Since these cells are an adherent cell line, they attach to the culture flask surface. Hence, the cells were regularly dislodged by a 50:50 enzyme solution of 0.25% trypsin mixed with 0.1% ethylenediamine tetra acetic acid disodium salt (EDTA) in order to divide the cells into new flasks. This process is known as trypsinization or passaging, meaning that every time cells are trypsinized they are given the next chronological passage

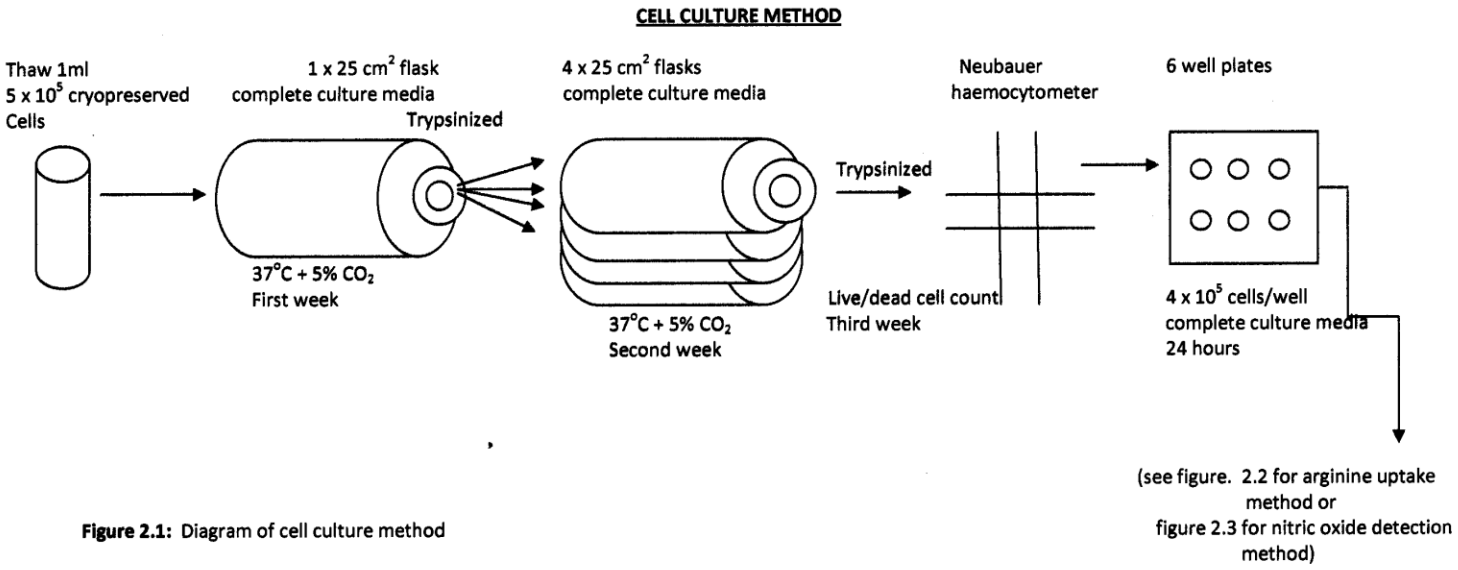


Figure 2.1: Diagram of cell culture method

number. Passage numbers are especially important in primary cell cultures with a limited lifespan, indicated by the number of possible passages before cell multiplication ceases. Cells were trypsinized once weekly when the monolayer of cells reached confluency. The culture medium was aspirated off and the flask rinsed with 5ml sterile phosphate buffered saline (PBS pH 7.4) (Sigma-Aldrich, Chemie, GmbH). The PBS rinse removed the FCS contained in the medium, which inhibits the enzymatic action of trypsin. Five milliliters of trypsin/EDTA solution was placed in the flask, just covering the cells, and were incubated for about five minutes at 37ºC. The EDTA in the trypsin solution is important as it chelates Ca^{2+} (calcium) in the medium, hence preventing the inhibitory action of Ca^{2+} on trypsin. After the five minute incubation, a firm tap of the culture flask against the palm of the hand dislodged the cells. The trypsin/EDTA solution containing the dislodged cells was poured into a 15ml conical polypropylene centrifuge tube (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA). The flask was rinsed with 10ml complete M199 culture medium, which was poured off and added to the 5ml trypsin/EDTA cell suspension in the tube. The cell suspension was centrifuged at 250g (Beckman model TJ6, Beckman Coulter, Midrand, SA) for five minutes. The supernatant was poured off carefully leaving the cell pellet behind in the tube. One milliliter of complete M199 culture medium was added to the cell pellet and cells were pipetted up and down three to four times with a 1ml sterile pipette to evenly disperse the cells. The cell suspension was topped up with 3ml complete M199 culture medium to a final volume of 4ml and cells were resuspended by inversion. One milliliter of the cell suspension was then added to each of four new flasks containing 24ml complete M199 culture medium pre-warmed to room temperature. The four new flasks were then incubated at 37ºC with 5% CO_2. The above procedure of sub-culturing into four new flasks was followed weekly to maintain cells in culture for the duration of the study.

2.2.2 EA.hy926 culturing

The EA.hy926 endothelial cells, at passage number 231, were shipped as a monolayer of cells in a $5cm^2$ flask completely filled with 25ml Dulbecco's Minimum Essential Medium (DMEM) supplemented with 4.5g L^{-1} glucose and 10% FCS. Upon arrival, the cells were immediately placed at 37ºC to recover from the travel trauma. After 48 hours, the 25ml complete culture

medium (DMEM supplemented with glucose, FCS and Pen/Strep) was aspirated and kept for re-use. The cells were rinsed with PBS and trypsinized as described for ECV_{304} cells in section 2.2.1 above, changing the passage number to 232. After centrifugation the cell pellet was resuspended in 5ml of the aspirated complete culture medium. As recommended by Prof. C-J Edgell who originally established the EA.hy926 cell line, in order to acclimatize the cells after the travel trauma, the cell suspension was divided into five new flasks (1ml each) and culture medium added as follows; (1) Four milliliter aspirated medium; (2) Three milliliter aspirated medium with 1ml freshly prepared DMEM supplemented with glucose and FCS; (3) Two milliliter aspirated medium with 1ml freshly prepared DMEM supplemented with glucose and FCS; (4) One milliliter aspirated medium with 1ml freshly prepared DMEM supplemented with glucose and FCS; (5) Five milliliter freshly prepared DMEM supplemented with glucose and FCS. One week later the flasks with sufficient growth of cells were trypsinized, divided into ten new flasks with fresh culture medium. After another week, pilot study flow cytometric analysis was done as described in section 2.1.2 above.

2.2.3 HUVEC culturing

Since primary cultures have special medium and sub-culturing requirements, the culture medium with supplements and the chemicals for sub-culturing were purchased from the cell suppliers (Clonetics®, Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA). A 1ml cryovial of frozen medium containing 5×10^5 HUVEC was received from Clonetics.

The 1ml cryovial of cells was quickly thawed in a water bath at 40ºC and added to 4ml of special culture medium containing Pen/Strep antibiotics, endothelial cell basal medium-2 (EBM-2) (Clonetics®, Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA) in a $5cm^2$ culture flask at room temperature (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA). The EBM-2 culture medium was supplemented with the following growth factors supplied as an endothelial growth medium-2 (EGM™-2) BulletKit: hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, FBS, hEGF and GA-1000 (Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA). Once the

contents of the EGM-2 BulletKit are added to the EBM-2 culture medium, the medium is referred to as complete culture medium. The cells were incubated at 37ºC with 5% CO_2 for 24 hours. The complete culture medium was replaced with fresh culture medium and cells were incubated for a week at 37ºC with 5% CO_2. The sub-culturing process differed from the ECV_{304} cells in that a ReagentPack™ (Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA) containing ready prepared trypsin/EDTA solution, trypsin neutralizing solution and (*N*-[2-Hydroxyl]piperazine)-N^1-[2-ethanesulfonic acid] (HEPES) buffered saline solution was used in the trypsinization process as supplied and recommended by Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA. The culture medium was aspirated and cells rinsed with 5ml room temperature HEPES solution. The HEPES solution was aspirated and the cells covered with 2ml trysin/EDTA solution for about five minutes. After a tap of the flask against the palm of the hand, the action of the enzyme solution was stopped by adding 4ml trypsin inhibitor solution (Lonza, Walkersville, MD, USA via Whitehead Scientific, Brackenfell, SA). The HUVEC were sub-cultured into four new $5cm^2$ flasks weekly. Only eight successive passages of HUVEC were completed before the cells ceased multiplying, which is normal for primary cells which naturally have a limited lifespan of a few weeks.

Hence, these cells were only used in studies to confirm that arginine uptake kinetics (see Chapters 3; 4 and 5) and NO production (see Chapter 4) is similar for both ECV_{304} and HUVEC.

2.3 Arginine uptake kinetics and nitric oxide determination (Figures 2.2 and 2.3).

(Arginine uptake method adapted from the transport assay as described by Gazzola *et al.*, 1981. *Analytical Chemistry* **115**:368-374).

After trypsinization of cells grown in flasks as described above in sections 2.2.1 and 2.2.2, a cell count was done using a Neubauer haemocytometer cell counter (Sigma-Aldrich, Chemie, GmbH) and trypan blue exclusion dye (Sigma-Aldrich, Chemie, GmbH). The trypan blue exclusion dye distinguishes between live and dead cells by entering dead cells through the

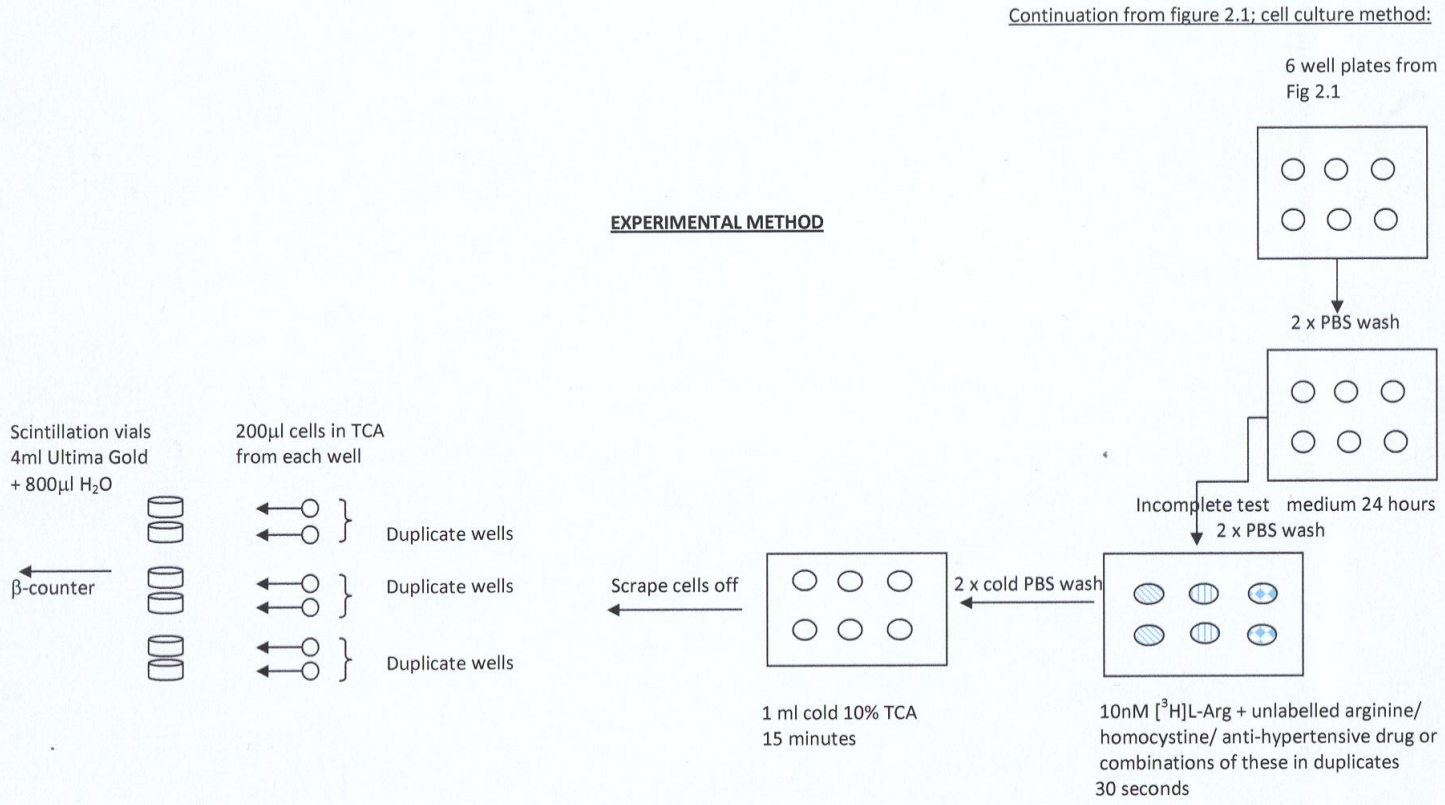


Figure: 2.2: Diagram of arginine uptake method

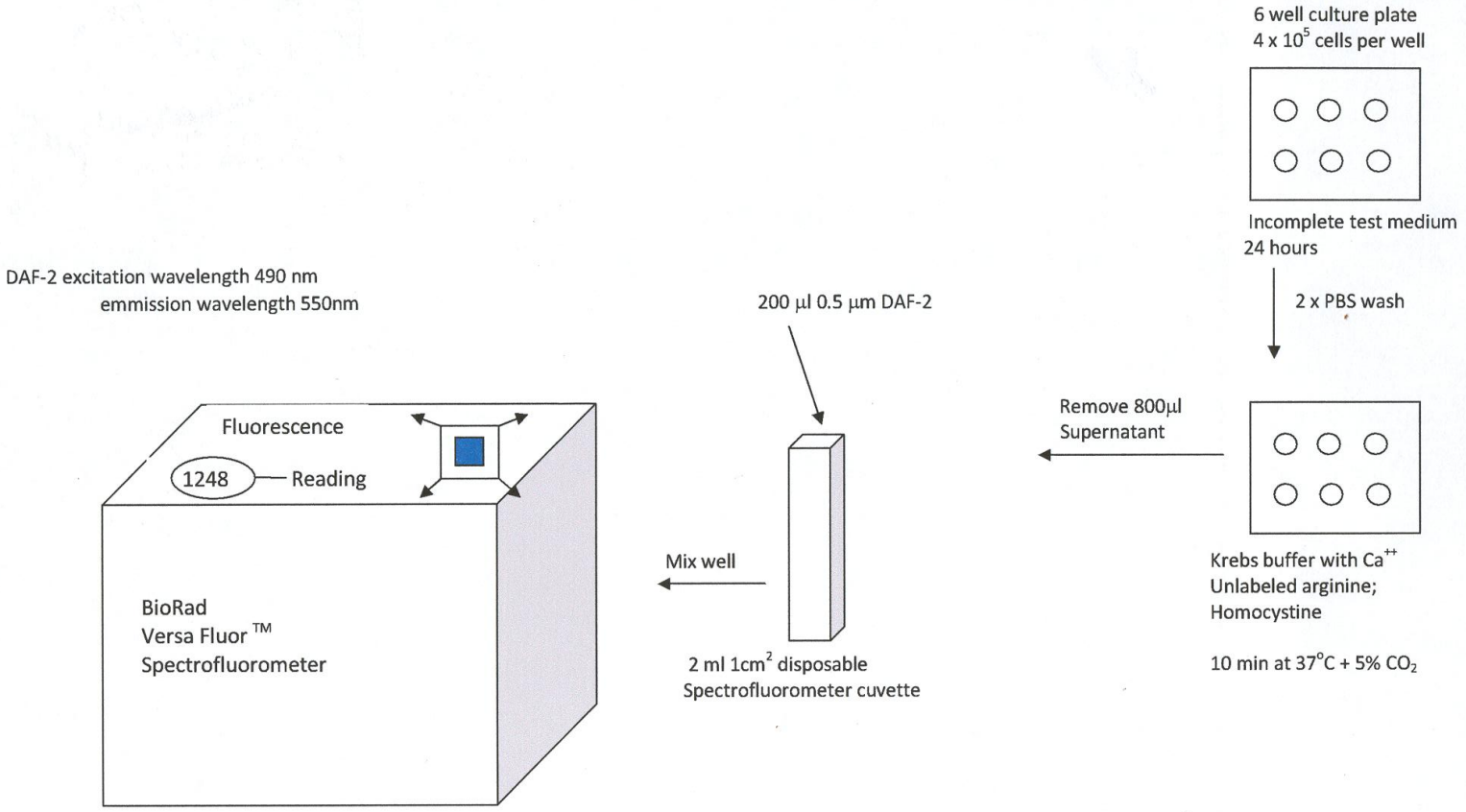


Fig 2.3: Diagram of nitric oxide detection method

dysfunctional cell membrane, staining the cells blue. The live cells appear clear and unstained through the microscope, since the live cell membranes are intact.

Four hundred thousand (4×10^5) live ECV_{304} cells (Chapters 3, 4 and 5) or HUVEC (Chapters 3, 4 and 5) were plated out into each well of six well cell culture plates (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA). The cells were plated in either 2ml M199 complete culture medium for ECV_{304} cells or 2ml EBM-2 complete culture medium for HUVEC. Cells were incubated for 24 hours in aerated humidified plastic containers, to prevent culture medium evaporation, at 37°C with 5% CO_2.

The 2ml M199 or EBM-2 complete culture medium was aspirated, the cells rinsed twice with 1ml PBS at room temperature and the complete culture medium of both the ECV_{304} and the HUVEC cells was replaced with 2ml custom made test medium consisting of M199 without arginine, without glutamine and with no FCS added to deplete the cells of arginine. Cells were incubated for another 24 hours at 37°C with 5% CO_2 (Sessa *et al.*, 1990). Before the addition of the test medium containing the radiolabelled arginine, the cell viability was determined again by using a Neubauer heamocytometer cell counter and trypan blue exlusion dye.

Standard M199 culture medium (Highveld Biologicals, Sandringham, Johannesburg, SA) contains 123nM each of vitamin B_6 pyridoxal and pyridoxine, 22.7nM folic acid, 342µM glutamine and 400µM arginine. Custom made M199 without arginine, glutamine, FCS, vitamin B_6 and folic acid (Highveld Biologicals, Sandringham, Johannesburg, SA) was obtained for the arginine uptake studies. I added the vitamins (Highveld Biologicals, Sandringham, Johannesburg, SA) to the custom made medium just before use, at concentrations of 123nM for each of the vitamins B_6 pyridoxal and pyridoxine, and 22.7nM of folic acid as per complete M199 culture medium. The custom made M199 without glutamine, arginine and FCS is hereafter referred to as test medium. Glutamine is usually added to culture medium at a concentration of 2mM to assist in cellular growth, but it was omitted from the test medium since it could influence my study by inhibiting intracellular arginine formation and subsequently NO production. Indeed, glutamine was shown to inhibit

arginine formation from citrulline in endothelial cells (Sessa *et al.,* 1990; Hecker, Sessa *et al.,* 1990; Hecker, Mitchell, Swierkosz *et al.,* 1990) and thereby decrease the production of NO (Mitchell, Hecker, Vane; 1990; Sessa *et al.,* 1990; Hecker, Sessa *et al.,* 1990; Hecker, Mitchell, Swierkosz *et al.,* 1990). I confirmed these findings in my own pilot studies using flow cytometric detection of DAF-2/DA as a sensitive indicator of intra-cellular NO in ECV_{304} cells. Indeed, I found that the presence of 2mM glutamine in M199 decreased NO production by about 35% (data not shown). Arginine was omitted from the test medium since I was studying the initial rate of arginine uptake and thus wanted the cells to be starved of arginine for 24 hours (Hecker, Sessa *et al.,* 1990; Sessa *et al.,* 1990; Mitchell, Hecker, Vane; 1990) prior to the addition of extracellular arginine. The FCS was omitted from the test medium since serum contains a variety of amino acids at varying concentrations which could influence the initial rate of arginine uptake and subsequent NO production. My pilot studies to determine flow cytometric detection of DAF-2/DA indicated that the presence of 10% FCS in M199 reduced NO production in ECV_{304} cells by about 9% (data not shown).

Due to the EBM-2 culture medium being copyright protected by Clonetics®, Lonza, Walkersville, MD, USA and information of the constituents thus not being available, no custom made EBM-2 medium without arginine and glutamine could be obtained. Since I wanted to compare arginine uptake in the immortalized ECV_{304} cell line to uptake in the primary HUVEC cells, I thought it best to change both cell types to the same test medium before adding arginine to the cells. Hence, as for ECV_{304} cells, the HUVEC cells were prepared in the custom made M199 test medium. Prior to addition of extracellular arginine, the 2ml custom made M199 test medium was aspirated and the cells were rinsed twice with 1ml PBS solution at room temperature.

From this stage the NO determination method differs from the arginine uptake method and is described in section 2.3.2 below and illustrated in figure 2.3.

2.3.1 **Arginine uptake methodology** (Figure 2.2).

For the arginine uptake studies, a total volume of 1ml of the freshly prepared arginine transport inhibitor solutions specific for the research question, and/or the radiolabelled tritiated arginine, L-[2,3,4-^{3}H] monohydrochloride arginine (1mCi (37MBq), Perkin Elmer, New England Nuclear, Boston, Mass), abbreviated L-[^{3}H]arginine (Chapters 3, 4 and 5), was added to the cells. The test solutions consisted of 10mM PBS solution containing 10nM L-[^{3}H] arginine and the specific concentration of the unlabelled amino acid (Chapters 3 and 4) or the drug (Chapter 5) in question (Sigma-Aldrich, Chemie, GmbH) for the particular experiment. The 10nM L-[^{3}H]arginine was used as a tracer molecule for the detection of arginine taken up by the cells. Test solutions at room temperature, were added while agitating the culture plate (Gazzola *et al.,* 1981; White & Christensen; 1982b; Sala *et al.,* 2002), to the cells for 30 seconds only, since my particular interest was the initial rate of arginine uptake by endothelial cells.

After 30 seconds (Gazzola *et al.,* 1981; White & Christensen; 1982b; Sala *et al.,* 2002), the 1ml radiolabelled test solution was aspirated and discarded in the radioactive liquid waste container. The cells were immediately washed twice with 1ml ice cold PBS to prevent further arginine uptake. The wash solution was aspirated and discarded in the radioactive liquid waste container.

One milliliter ice cold 10% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) in water was placed directly onto the cells in each of the wells for a minimum of 15 minutes (Gazzola *et al.,* 1981), to fix the cells. The TCA precipitates the cellular proteins which results in cessation of all cell functions, thereby fixing the cells in their present state (McDonald, Rouhani *et al.,* 1997).

After 30 minutes the cells were scraped off with a cell scraper (Costar, The Scientific Group Adcock Ingram, Midrand, Gauteng, SA)). Two hundred microliter cell suspension in 10% TCA from each well was added to a scintillation vial containing 4ml Ultima Gold scintillation fluid (Separation Scientific, Honeydew, Johannesburg, SA) and 800µl filter sterilized water.

Samples were analyzed on a β-liquid scintillation analyzer (Packard 1600CA Tri-Carb, Canberra Packard, Meriden, IL, USA) for five minutes.

The counts obtained were captured on an MSExcel spreadsheet and corrected for uptake time. After correction for the arginine dilution factor, the rate of arginine uptake by the cells was calculated to generate Michaelis-Menten, Lineweaver-Burk and Eadie Hoffstee graphs. The rate (*v*) of arginine uptake by the cells was expressed as nM arginine per 4 x 10^5 cells per minute. To calculate the kinetic constants of cationic amino acid uptake into endothelial cells, the raw data was used directly in non-linear models to determine the kinetic constants using methods previously described by Malo & Berteloot (1991), of which a detailed description follows in chapter three.

In this study, it was assumed that the uptake of ^{3}H-labelled or unlabelled arginine was identical. In preliminary experiments I determined the linearity of labelled arginine uptake with time and concentration. The uptake of the labelled arginine in the presence of unlabelled arginine with time was also determined (author's unpublished data).

2.3.2 Nitric oxide determination (Figure 2.3).

The ECV_{304} cells and HUVEC were cultured as described above in sections 2.2.1 and 2.2.3 respectively.

For the NO determination part of this study, a diaminofluoresceine (DAF), developed specifically for the direct detection of NO with the advantages of being specific, sensitive and with a simple protocol, yielding a bright green fluorescence (Kojima *et al.,* 1998), was used. I adapted my methodology from methods previously described for the intracellular detection of NO by flow cytometry (Strijdom *et al.,* 2004; 2005), and by confocal microscopy (Peluffo; 2007) using the DAF-2 diacetyl ester [DAF-2(DA)] form of DAF which is cell membrane permeable, and for the method for extracellular detection of NO by spectrofluorimetry (Nakatsubo *et al.,* 1998; Leikert *et al.,* 2001), using the free acid form of DAF-2 , which is not

membrane permeable, to detect NO in solutions, buffers and cell culture medium (Sigma-Aldrich, Chemie, GmbH).

2.3.2.1 Nitric oxide standard curve

A NO generator, diethylamine NONOate sodium salt hydrate (DEA/NO) (Sigma-Aldrich, Chemie, GmbH) dissolved in double glass distilled, deionised water, instead of NO producing cells was used to construct a standard curve to ensure the methodology was optimized and NO specific. Eight hundred microliter of DEA/NO at concentrations ranging from 0μM with increments of 10 up to 100μM, was mixed with 200μl fluorescent DAF-2 (Sigma-Aldrich, Chemie, GmbH) NO specific dye at a final concentration of 0.5μM in Krebs Henseleit buffer pH 7.4 (Appendix A). Spectrofluorometer readings were recorded immediately and a standard curve generated in Microsoft Office 2008 Excel.

2.3.2.2 Cellular nitric oxide generation

The cellular NO detection methodology is as described above in section 2.3 up to the stage where the M199 test medium was aspirated and the cells washed twice with one ml PBS at room temperature.

Instead of adding 1ml radiolabelled arginine solutions in PBS to the cells, the M199 test medium was replaced with 1ml solutions of a range of unlabelled arginine or homocystine or antihypertensive drug or combinations of these (Sigma-Aldrich, Chemie, GmbH) in Krebs Henseleit buffer pH 7.4 (Appendix A), in duplicate wells. The cells were then incubated for ten minutes at 37 ^{o}C with 5% $CO_{2.}$ Krebs Henseleit buffer with an increased Ca^{2+} concentration, to obtain optimal results for the fluorescent DAF-2 NO sensitive dye, was used as recommended by the suppliers (personal e-mail communication) (Sigma-Aldrich, Chemie, GmbH).

Eight hundred microliter aliquots of the amino acid (arginine or homocystine) or antihypertensive drug solutions were removed from the culture wells, and placed in disposable

2ml 1cm^2 spectrofluorometer cuvettes (BioRad, Rosebank, Johannesburg, SA). Two hundred microliter of the NO sensitive fluorescent dye DAF-2 at a final concentration of 0.5μM in Krebs Henseleit buffer pH 7.4 was added to each 800μl solution aliquot. These test samples were mixed well and read immediately, for times up to thirty minutes at room temperature on a medium gain setting at excitation and emission wavelengths of 490 and 550nm respectively on a VersaFluor^{TM} spectrofluorometer (BioRad, Rosebank, Johannesburg, SA). Spectrofluorometer readings were recorded for each sample at each time interval and fluorescence detection curves were generated.

2.4 Data analysis

The data was analysed using GraphPad Prism® Version 5, GraphPad Software Inc, La Jolla, California, USA. Data and statistical analysis related to the arginine uptake kinetics is described and discussed in detail in Chapter 3 and is used in the subsequent Chapter 4 and Chapter 5. Nitric oxide production and the effects of homocystine and antihypertensive drugs on NO production are discussed in detail in Chapter 4 and Chapter 5 respectively.

CHAPTER 3

Modelling of cellular arginine uptake by more than one transporter

3.0 Abstract

Determining kinetic constants of arginine uptake by endothelial cells mediated by more than one transporter from linearization of data as Eadie-Hofstee plots, or modelling which does not include the concentration of trace radiolabelled amino acid used to measure uptake may not be correct. Initial rate of uptake of trace L-[^{3}H]arginine by HUVEC and ECV_{304} cells in the presence of a range of unlabelled arginine and modifiers, was used in non-linear models to calculate constants of arginine uptake using GraphPad Prism. Theoretical plots of uptake derived from constants determined from Eadie-Hofstee graphs overestimated uptake whereas those from the non-linear modelling approach agreed with experimental data. The contribution of uptake by individual transporters could be modelled and showed that leucine inhibited the individual transporters differently and not necessarily competitively. *N*-ethylmaleimide inhibited only y^+ transport and BCH may be a selective inhibitor of y^+L transport. The absence of sodium decreased arginine uptake by y^+L transport and decreased the K_m', whereas decreasing sodium decreased arginine uptake by y^+ transport without affecting the K_m'. The non-linear modelling approach using raw data, avoided the errors inherent in methods deriving constants from the linearization of the uptake processes following Michaelian kinetics. This study provides explanations for discrepancies in the literature and suggests that a non-linear modelling approach better characterises the kinetics of amino acid uptake into cells by more than one transporter.

3.1 Introduction

The semi-essential, cationic amino acid, L-arginine plays an important role in cellular function and metabolism, and is the known precursor of the vasodilator nitric oxide (NO). Arginine potentiates the release of NO from endothelial cells grown in arginine depleted medium, thereby regulating NO production (Mitchell, Hecker, Änggård *et al*. 1990). Arginine may limit

NO production in subjects with normal blood pressures, but not in patients with hypertension (Panza, Casino, Badar *et al*. 1993). Indeed, fasting plasma arginine concentrations are elevated in patients with hypertension (Penttinen *et al*. 2000; Moss *et al*. 2004; Perticone *et al*. 2005; Naidoo *et al*. 2009), and yet supplemental arginine can decrease blood pressure in such patients (Siani *et al.* 2000; Ast *et al*. 2010). One explanation for these data may be that arginine uptake into cells was impaired and therefore would not be available for NO production. In support of such a hypothesis, studies have shown that cellular uptake of arginine is decreased in lymphocytes from patients with hypertension and individuals genetically predisposed to developing hypertension (Schlaich *et al*. 2004). However, elucidating the kinetics of arginine uptake into endothelial cells is fundamental to determine whether arginine uptake is indeed impaired in these cells.

Studies to date have determined that cationic amino acid uptake into endothelial cells is mediated by the high affinity/low rate y^+L transporter and the low affinity/high rate y^+ transporter. Uptake mediated by the y^+ transporter is sodium-independent, pH-insensitive, stereoselective and is inhibited by N-ethylmaleimide (NEM) and certain neutral amino acids (Devés & Boyd, 1998). Transport of arginine and other cationic amino acids by the y^+L transporter is sodium-independent and in the presence of sodium, neutral amino acids such as methionine, glutamine and leucine, may also be transported with high affinity (Devés & Boyd, 1998; Mann *et al*. 2003; Bröer, 2008). Although recent studies have described the activity and properties of transporter gene products CAT-1, CAT-2A and CAT-2B (y^+), and 4F2hc/y^+LAT1 (SLC3A2/SLC7A7) and 4F2hc/y^+LAT2 (SLC3A2/SLC7A6) (y^+L) (Closs, Basha *et al.* 1997; Mann *et al*. 2003, Bröer, 2008), ascribing and reconciling the activities of these gene products to earlier studies which only measured activity, can be difficult, as the use of different cell types and experimental approaches used when determining uptake rates has complicated direct comparisons of the data.

Most studies determining the uptake of radiolabelled amino acids have assumed Michaelis-Menten kinetics and have calculated constants from Lineweaver-Burk reciprocal plots, Eadie-Hofstee plots and non-linear modelling (Christensen & Antonioli; 1969; White & Christensen; 1982b; Closs, Lyons *et al.*, 1993; Moss *et al.*, 2004, Brunini *et al*., 2006). Determining the

kinetics, the relative contribution and physiological importance of individual transporters in cells expressing more than one transporter, may be complicated (Rotmann *et al*., 2007) and thus may lead to discordant results. Indeed, early *in vitro* kinetic uptake studies that ascribed uptake to only y^+ transport (Christensen *et al*., 1994) appear to have overlooked the contribution of y^+L transport (Devés *et al.*, 1992). Moreover, when determining kinetic parameters of individual cationic amino acid transporters operating simultaneously in a cell, studies have made various assumptions. In particular, studies

a) have measured uptake of trace labelled substrate in the presence of unlabelled substrate, without factoring in the relative concentrations of these when calculating the kinetic parameters (Sobrevia *et al*., 1995; Durante *et al*., 1996; Dall'Asta *et al*., 2000; Casanello & Sobrevia; 2002; Hardy & May; 2002; Arancibia-Garavilla *et al*., 2003). In this regard, the rate of uptake of labelled amino acid in the presence of unlabelled amino acid is not linear (Devés *et al*., 1992).
b) in which radiolabelled tracers have been used, the subtraction of non-specific uptake and then using Eadie-Hofstee linearization of data is not suitable to determine the existence of more than one transporter of uptake (Malo & Berteloot; 1991) since this may lead to overestimation of the kinetic parameters obtained, as also mentioned later on in section 3.3.2.
c) determining relative uptake rates at specific concentrations of the amino acids and their inhibitors (Arancibia-Garavilla *et al.*, 2003; Signorello *et al*., 2003; Rotoli *et al*., 2005) may not infer that such rates apply at other substrate and/or inhibitor concentrations.
d) have measured total uptake, then inhibited one of the transporters to determine the residual activity of the other transporter by subtraction. This assumes a linear and additive uptake independent of trace labelled and unlabelled substrate concentrations (Mendes-Ribeiro *et al*. 1999; Ayuk *et al*. 2002, Brunini *et al*. 2006; Rotmann *et al*. 2007).
e) calculated constants from models of competitive inhibition of cationic amino acid uptake by structurally dissimilar neutral amino acids, such as leucine and glutamine (Devés *et al*., 1992; 1993), without determining whether non-competitive or other forms of inhibition significantly improved the fit with the experimental data.

f) determining inhibitor concentrations at which uptake was inhibited by 50% (I_{50}) (Hardy & May; 2002; Rotoli *et al*., 2005), have also only modelled competitive inhibition (Cheng & Prusoff; 1973) by these structurally dissimilar neutral amino acids (Angelo *et al.*, 1996; Dall'Asta *et al*., 2000).

To address these issues, I modelled the uptake of the cationic amino acid, arginine into cells using the general non-linear approach of Malo & Berteloot (1991). This approach 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, no assumptions are made regarding the type of inhibition and the concentrations of inhibitors (or activators) included in the model. As the model was additive, the theoretical contribution of uptake by each transporter could be modelled.

I tested this approach by modelling initial rate kinetics of arginine uptake by two transporters into ECV_{304} cells, over a range of substrate and inhibitor concentrations, and reproduced the approach in primary endothelial cells (HUVEC). Importantly, the results explain discrepancies in the literature when determining kinetic parameters of cationic amino acid transport by more than one transporter.

3.2 Experimental methodology

The culture methods for ECV_{304} and HUVEC are described in sections 2.2.1 and 2.2.3 respectively of the Materials and Methods Chapter 2 and illustrated in figure 2.1. The methods for the determination of arginine uptake are described in section 2.3 and illustrated in figure 2.2 of the Materials and Methods Chapter 2.

For the transport inhibitor studies, test medium were removed and the cells were washed twice with 1ml PBS as described in section 2.3 of Chapter 2. Thereafter, cells were incubated with 1ml of either one of NEM, BCH, leucine or sodium.

The NEM or BCH inhibitor was added to the cells prior to the 30 second 10nM L-[^{3}H]arginine incubation as follows:

- cells were incubated for 10 minutes at room temperature with 1ml solution of 0.2mM NEM in PBS. After the 10 minutes, the reaction was stopped by adding 10µl of a 10mM solution of 2-mercaptoethanol to the NEM solution, and cells were incubated at room temperature for another 10 minutes.
- cells were incubated for 60 minutes at room temperature with 1ml solution of 30mM BCH in PBS.

In both of the above, the cells were then washed twice with 1ml PBS after these incubation periods in preparation for the 30 second incubation with L-[^{3}H]arginine as described in section 2.3.1 and illustrated in figure 2.2 of Chapter 2.

The leucine inhibition experiments and the sodium dependency experiments did not require an extra incubation step as described above for NEM and BCH;

- in the leucine experiments, the different concentrations (0.033, 0.067, 0.1, 0.2, 0.5mM) of leucine in PBS was added to the L-[^{3}H]arginine solution and the cells were incubated in this combined solution for 30 seconds.
- in the sodium dependency experiments, cells were incubated in either sodium depleted PBS with L-[^{3}H]arginine, or in PBS containing varied sodium concentrations (26mM, 157mM) with L-[^{3}H]arginine for 30 seconds.

After the 30 second incubation periods, the respective L-[^{3}H]arginine solutions were removed and discarded and methodology as described in section 2.3.1 (Figure 2.2) was followed.

3.2.1 Determination of uptake kinetic constants

In this study, I assumed that the uptake of ^{3}H-labelled or unlabelled arginine was identical.
In initial experiments, I determined the rate of arginine uptake into ECV_{304} cells at 10, 30 and 60 seconds in order to determine the time at which the rate of uptake was most reproducible. The time of 30 seconds was then used for all subsequent experiments. In other preliminary experiments I determined the linearity of labelled arginine uptake with time and concentration. Further, I also determined the uptake of the labelled arginine in the presence of unlabelled arginine, with time. Uptake of radiolabelled arginine by the cells was expressed as nM per

$4x10^5$ cells per minute, and these data was used directly in non-linear models to determine kinetic constants using methods described previously (Malo & Berteloot; 1991).

After correcting for dilution of the isotope (Coons *et al.,* 1995), rate *vs* substrate plots were construceted assuming an additive model for two transporters following Michaelis-Menten kinetics and the data was linearized as Eadie-Hofstee plots and/or Lineweaver-Burk plots which, if curvilinear may suggest the presence of two independent transporters. However, when using radiolabelled tracers, the latter approach, particularly when subtracting non-specific uptake from total uptake, results in loss of statistical information (Malo & Berteloot; 1991).

Hence, the rate of uptake (v_T) of undiluted tracer (T) by a single transporter, in the presence of unlabelled substrate [S], where the maximum velocity (rate), (V_{max}) indicates the maximum concentration of arginine [S] taken up per unit of time, and the Michaelis-Menten constant, (K_m) indicates the arginine concentration [S] at which the velocity (rate) of arginine uptake is half the maximum velocity (V_{max}), may be described by Michaelis-Menten kinetics (Malo & Berteloot; 1991) as follows:

$$v_T = \frac{V_{max}*[T]}{(K_m + [T] + [S])} \qquad \text{Eq 1.}$$

If two independent transporters (denoted by the subscripts *a* and *b*) were present, in the presence of unlabelled substrate and diffusion ($k_D*[T]$), where k_D is the diffusion rate constant, the rate of uptake of the labelled substrate was additive:

$$v_T = \frac{V_{maxa}*[T]}{(K_{ma} + [T] + [S])} + \frac{V_{maxb}*[T]}{(K_{mb} + [T] + [S])} + k_D*[T] \qquad \text{Eq 2.}$$

3.2.2 Statistical Analysis

Equations 1 & 2 (above) were used to model the uptake of the 10nM labelled arginine in the presence of a range of concentrations of unlabelled arginine and various inhibitors. The maximal rates of uptake (V_{maxa}, V_{maxb}) and the Michaelis-Menten constants (K_{ma} and K_{mb}) were adjusted using non-linear regression analysis (GraphPad Prism®Version 5, GraphPad Software Inc, La Jolla, California, USA) to best fit the model to the experimental data.

Models of one versus two transporter uptake were tested and it was determined whether including a diffusion component improved the fit (Eq. 2). As the rate of uptake was fastest at low substrate concentrations and slowest at high substrate concentrations respectively, the curve was fitted by minimizing the sum of squares of relative distances ($1/Y^2$ weighting to restore equal weighting to all points in the curve) of the data from the curve. Further, after checking for outliers, a D'Agostino and Pearson normality test ($p>0.20$) was used to ensure that residuals were randomly distributed across the substrate concentrations. A robust fit of data which is less sensitive to outliers was also used. V_{maxa}, V_{maxb}, K_{ma} and K_{mb} were reported as mean ± SEM (standard error of the mean) or mean ± SD (standard deviation) as indicated.

Models of one versus two transporter uptake were tested and it was determined whether the inclusion of a diffusion component improved the fit (Eq. 2). In the presence of inhibitors, the type of inhibition by leucine was elucidated by determining whether either V_{max}' and/or K_m' and the ratio of K_m'/V_{max}' remained constant. The inhibition constants (K_i) were then calculated for both transporters (Krupyanko; 2007). In addition, using arginine uptake corrected for dilution of the isotope, an additive Michaelis-Menten equation (Eq. 3) was used to confirm the kinetic constant values.

$$v = \frac{V_{maxa}[S]}{K_{ma} + [S]} + \frac{V_{maxb}[S]}{K_{mb} + [S]} \qquad \text{Eq.3}$$

Further, as the results obtained for these constants were consistent with those reported in the literature (Devés & Boyd; 1998; Mann *et al*., 2003), transport by the higher affinity/lower rate

transporter (denoted by subscript '*a*' when K_{ma}, V_{maxa} and K_{ia} were reported) was also referred to as the y^+L transporter. Similarly, the lower affinity/faster rate transporter (denoted by subscript '*b*' when K_{mb}, V_{maxb} and K_{ib} were reported) was referred to as the y^+ transporter.

3.3 Results

3.3.1 Preliminary results

Arginine uptake into ECV_{304} was linear up to 50nM L-[^{3}H]arginine (Figure 3.1A) and for up to 5 minutes (Figure 3.1B). Initial experiments found 30 seconds was the earliest time when the rate could be reproducibly measured under the experimental conditions used (data not shown). In the presence of unlabelled arginine, the rate of uptake of 10nM [^{3}H]-labelled L-arginine decreased in an exponential manner over 5 minutes and was dependent on the concentration of unlabelled arginine (Figure 3.1C). At 30 seconds, the nonlinear dependence of the rate of 10nM L-[^{3}H]arginine uptake upon the concentration of unlabelled arginine was shown for both ECV_{304} (Figure 3.2A) and HUVEC (Figure 3.3A) respectively.

3.3.2 Arginine uptake by ECV_{304} and HUVEC

Kinetic constants, derived from dilution corrected linear transformed plot intercepts, were used as preliminary estimates in the non-linear additive models of uptake (Malo & Berteloot; 1991). For both ECV_{304} (Figure 3.2A) and HUVEC (Figure 3.3A), after excluding outliers and ensuring residuals between experimental data and the model fit were normally distributed within the range of unlabelled arginine tested (Figure 3.2B and 3.3B), a model of uptake by two transporters was preferred over that of a single transporter, irrespective of whether the models included a diffusion component ($p<0.0001$; data not shown). The two transporter model was also preferred to a single transporter model with *n* substrate binding sites and with diffusion (see Eq 3 in Chenu & Berteloot; 1993; data not shown). Furthermore, the Eadie-Hofstee (Figure 3.4A and 3.5A) and Lineweaver-Burk plots (Figure 3.4B and 3.5B) for both ECV_{304} and HUVEC respectively were curvilinear, hence supporting a model of uptake by two transporters.

In both ECV_{304} and HUVEC, the kinetics program used indicated that an uptake model of two transporters without diffusion was preferred, although the difference between the model with or without diffusion was not significant (Table 3.1). For both cell types, models including diffusion, were found to have large errors (SEM) for the estimates of the kinetic constants (K_{mb} and the diffusion constant 'k_D'), with wide confidence intervals for the diffusion constant (Table 3.1), supporting the indication that the model without diffusion was correct. Furthermore, a robust fit (less sensitive to outliers) of the data, found the diffusion parameter was different to that calculated by the least squares fit method, further indicating that the model including diffusion was not stable when fitting this model to the data. Finally, the contribution by diffusion of 10nM L-[^{3}H]arginine used in these experiments, to the total uptake was small (k_D*0.00001mM; Eq 2). Therefore, for subsequent experiments the diffusion component was excluded.

The V_{maxa} (y^+L transport) calculated for the ECV_{304} was similar to that of the HUVEC (p=0.34) whereas the V_{maxb} (y^+ transport) was higher for HUVEC compared to ECV_{304} (p=0.019). ECV_{304} affinity constants for both transporters were similar to those for HUVEC (K_{ma} and K_{mb}; ns; Table 3.1; Figures 3.6A and 3.6B). The relative maximum uptake (V_{maxb}/V_{maxa}) of the low affinity/high rate transporter (y^+) relative to the high affinity/low rate (y^+L) transporter was 3.6 in ECV_{304} and 8.8 in HUVEC respectively. The kinetic parameters determined by the non - linear modelling approach were similar to those determined using a model of Michaelis-Menten uptake with two transporters, after correcting uptake for dilution of the isotope (Figure 3.6A for ECV_{304} and Figure 3.6B for HUVEC).

The additive model approach allowed for the theoretical contribution of uptake by the individual transporters into ECV_{304} (Figure 3.6A) and HUVEC (Figure 3.6B) respectively. Theoretical total uptake calculated from the kinetic constants, derived from dilution corrected linear transformed plot intercepts (Eadie Hofstee plots; Table 3.1), significantly overestimated the experimental data, whereas theoretical uptake calculated from the non-linear modelling

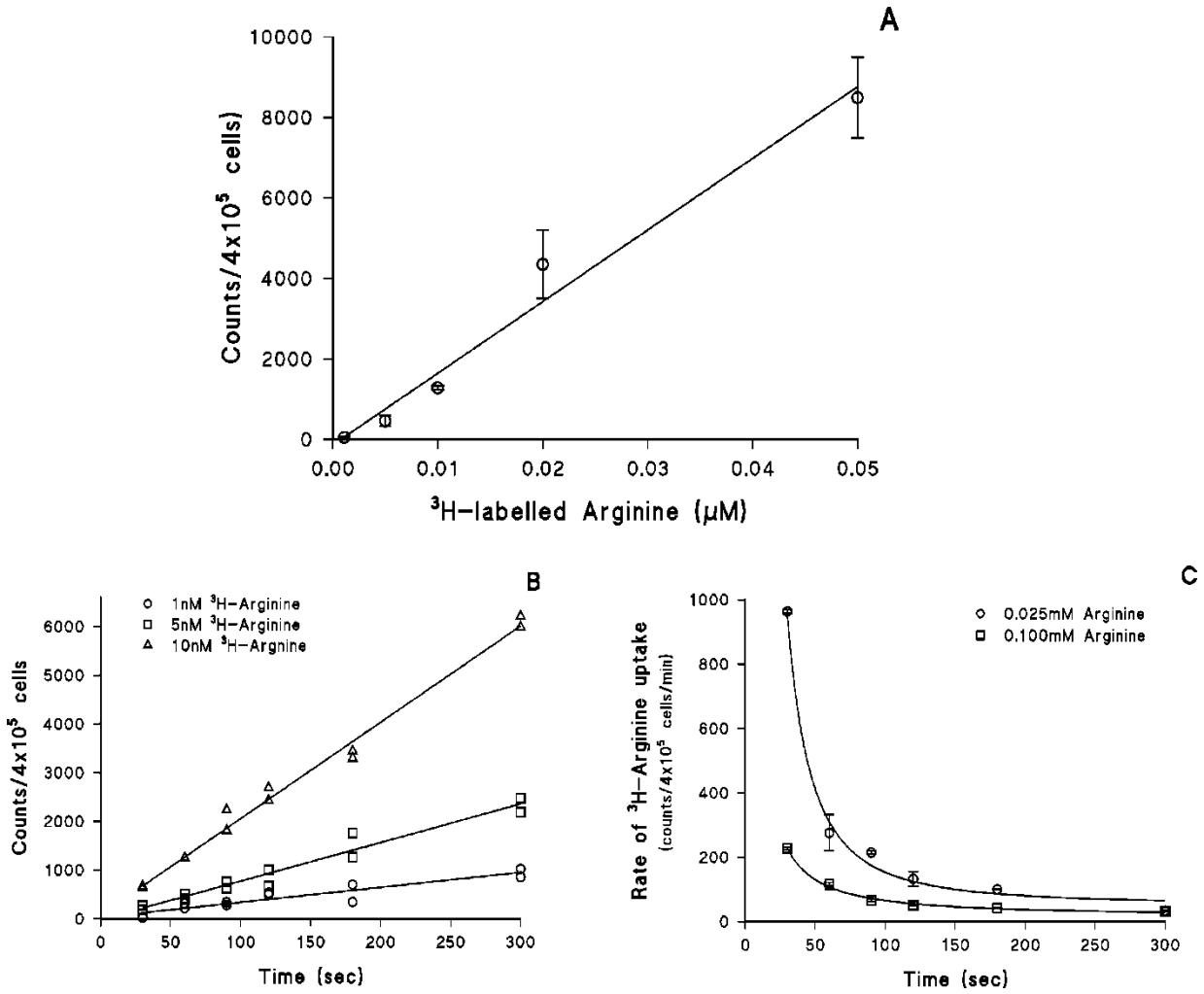


Figure 3.1 Uptake of labelled arginine by ECV_{304} showing: A. Uptake with concentration of L-[^{3}H]arginine (data as mean±SD from triplicate experiments); B. Uptake over time with concentration of L-[^{3}H]arginine; C. The non-linear decrease in the rate of 10nM L-[^{3}H]arginine uptake in the presence of 0.025 and 0.100mM unlabelled arginine with time. Experimental details have been described in the Materials and Methods section except that uptake was measured for up to 300 seconds before the reaction was stopped. Data for HUVEC was similar (data not shown).

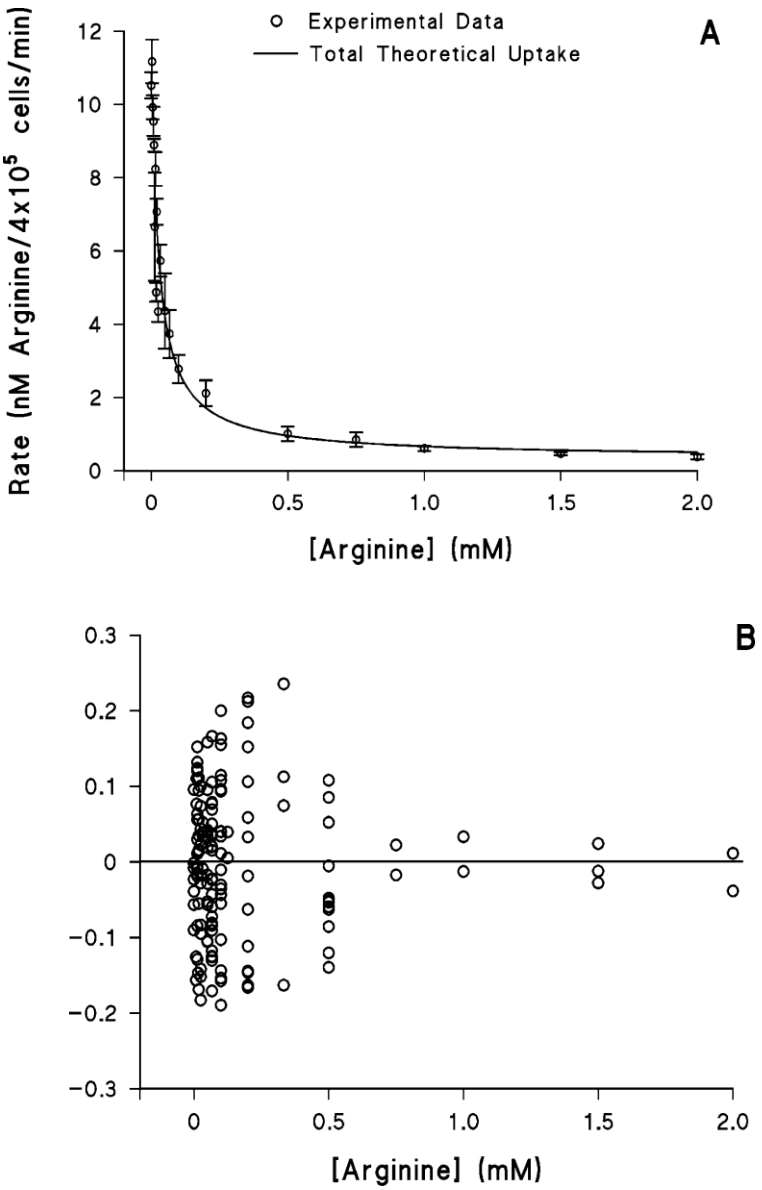


Figure 3.2 Data used to determine the kinetic constants of arginine uptake by non-linear modelling for ECV_{304}. A. Experimental uptake (as mean±SD from 10 experiments) of 10nM L-[^{3}H]arginine in the presence of increasing concentrations of unlabelled arginine was used with Eq. 2 to determine the kinetic constants (as described in sections 3.2.1 and 3.2.2). The curve is the theoretical uptake derived from these constants (see Table 3.1). B. Residuals for graph A. A D'Agostino and Pearson omnibus K2, p=0.383, indicated a lack of bias for the differences between experimental arginine uptake and the theoretical model contributions.

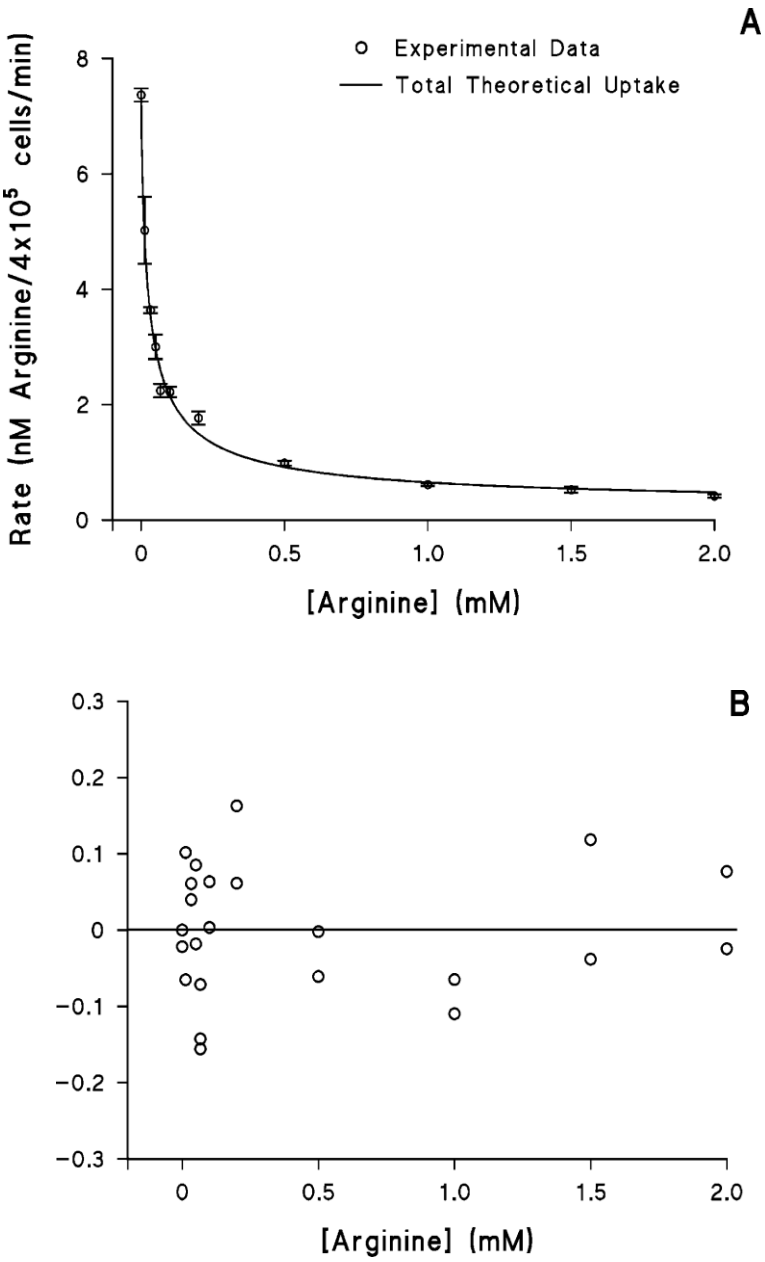


Figure 3.3 As for Figure 3.2 showing the data used to determine the kinetic constants of arginine uptake by non-linear modelling for HUVEC. A. Experimental uptake (as mean±SD from duplicate experiments) of 10nM L-[^{3}H]arginine in the presence of increasing concentrations of unlabelled arginine was used with Eq. 2 to determine the kinetic constants (as described in sections 3.2.1 3.2.2). B. Residuals for graph A. A D'Agostino and Pearson omnibus K2, p=0.858, indicated a lack of bias for the differences between experimental arginine uptake and the theoretical model contributions.

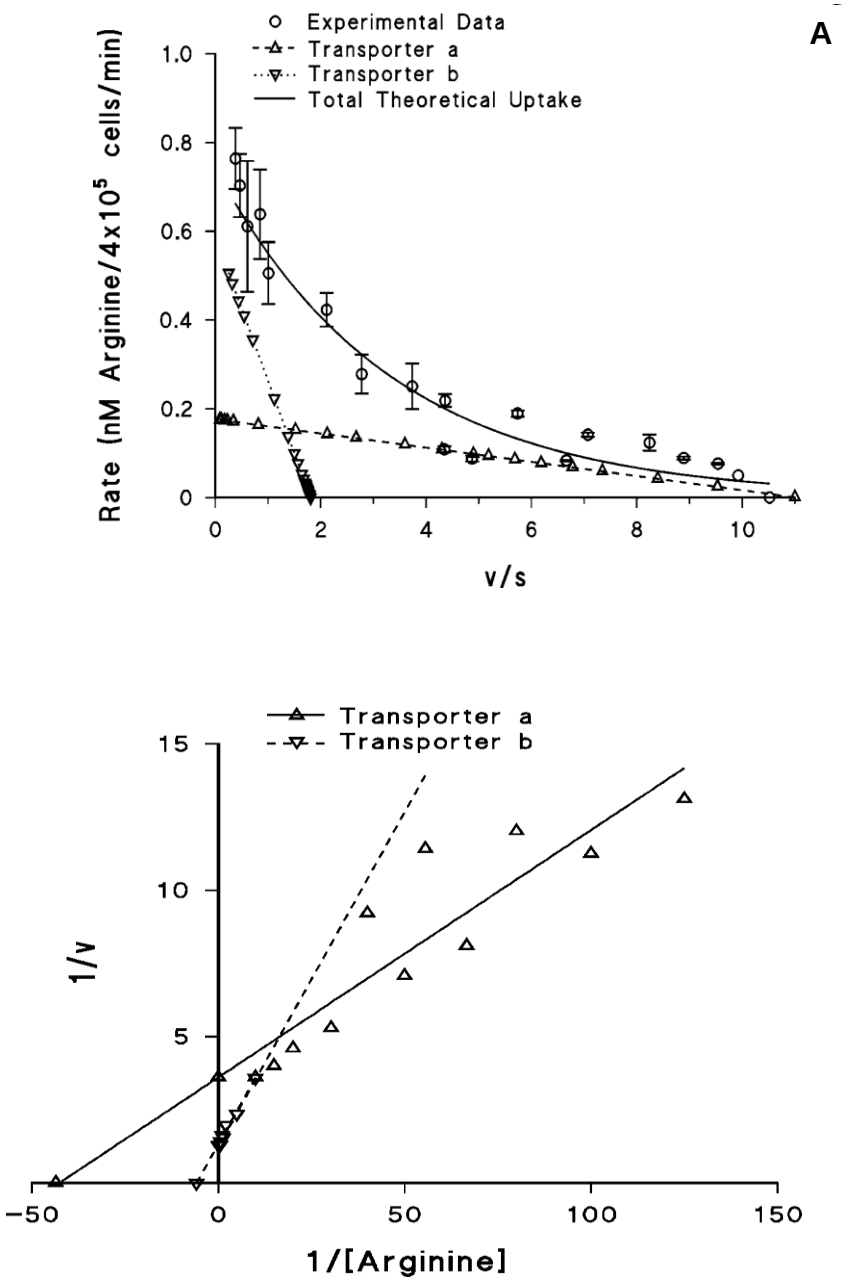


Figure 3.4 Data used to determine the kinetic constants of arginine uptake by non-linear modelling for ECV_{304}. A. Eadie-Hofstee plots calculated from dilution corrected data from Figure 3.2A showing the close agreement between total experimental uptake (mean±SD) and total theoretical uptake calculated from the derived constants of the non-linear modelling in Table 3.1. The straight lines are the theoretical plots (derived from the constants of non-linear modelling in Table 3.1) for each transporter to illustrate the differences between these lines and the theoretical curve of total transport. B. Data as in Figure 3.4A illustrated as Lineweaver-Burk plots.

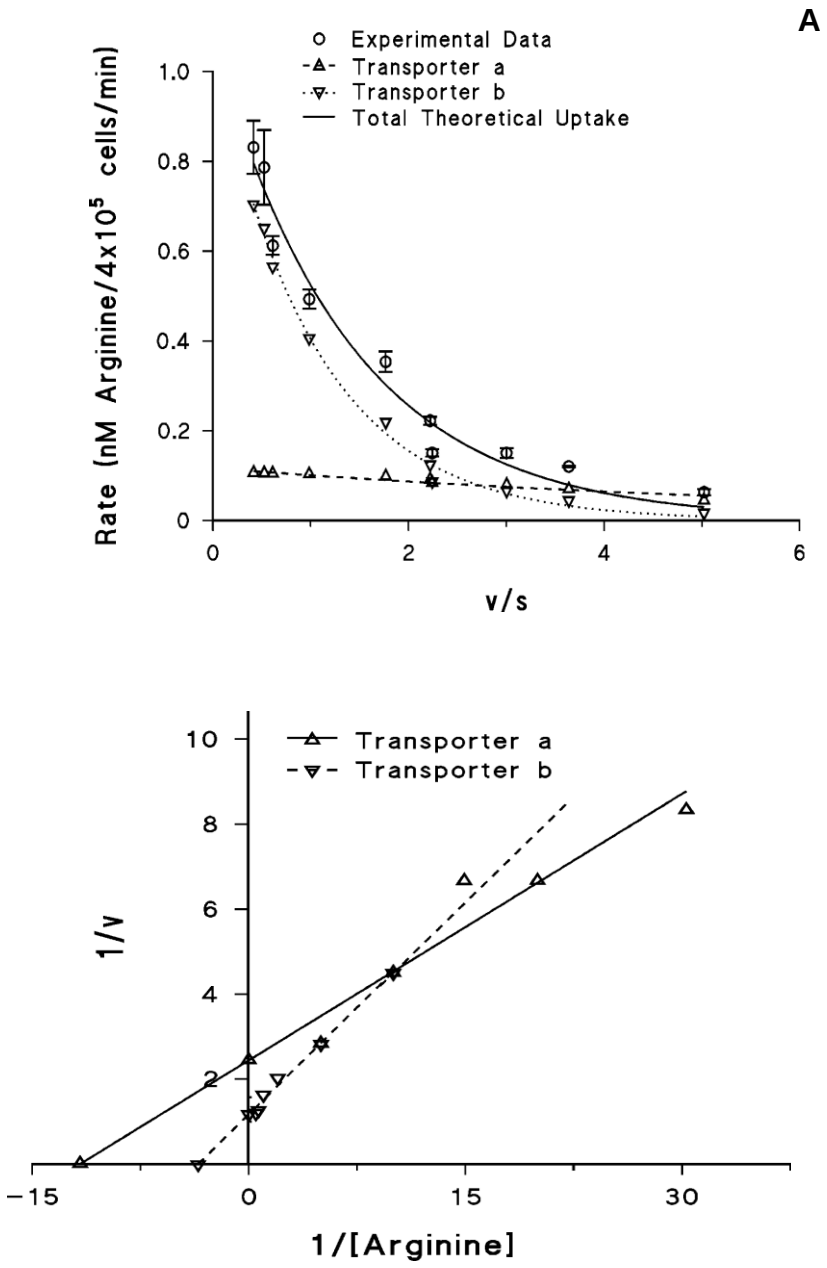


Figure 3.5 As for Figure 3.4 showing the data used to determine the kinetic constants of arginine uptake by non-linear modelling for HUVEC. A**.** Eadie-Hofstee plots calculated from dilution corrected data from Figure 3.3A showing the close agreement between total experimental uptake (mean±SD) and total theoretical uptake calculated from the derived constants of the non-linear modelling in Table 3.1. The straight lines are the theoretical plots (derived from the constants of non-linear modelling in Table 3.1) for each transporter to illustrate the differences between these lines and the theoretical curve of total transport. B. Data as in Figure 3.5A illustrated as Lineweaver-Burk plots.

Table 3.1 Comparison of kinetic constants obtained from Eadie-Hofstee plots (from dilution corrected data) and non-linear models (using raw data) of arginine uptake by ECV_{304} and HUVEC. Results are the mean±SEM from ten experiments in ECV cells and duplicates for HUVEC with the robust value (less sensitive to outlier data) in brackets (see Materials and Methods). Significant differences: * $p<0.05$, ** $p<0.005$, *** $p<0.0005$: Eadie-Hofstee plot *vs* non-linear model without diffusion for each cell type; † $p<0.05$: ECV_{304} *vs* HUVEC without diffusion. Differences for a model without diffusion *vs* with diffusion were not significant for all parameters.

	ECV			**HUVEC**		
	Eadie Hofstee plot	**Model without diffusion[1]**	**Model with diffusion**	**Eadie Hofstee plot**	**Model without diffusion[2]**	**Model with diffusion**
V_{maxa}	0.294±0.042*	0.176±0.026	0.136±0.034	0.225±0.019**	0.106±0.026	0.091±0.037
(nM arginine/4x10^5 cells/min)		(0.243)	(0.235)		(0.122)	(0.120)
K_{ma} (mM)	0.024±0.006	0.016±0.002	0.013±0.003	0.030±0.005	0.018±0.004	0.016±0.005
		(0.021)	(0.021)		(0.020)	(0.020)
V_{maxb}	0.768±0.035***	0.589±0.028	0.459±0.054	1.038±0.09	0.933±0.077†	0.646±0.259
(nM arginine/4x10^5 cells/min)		(0.558)	(0.407)		(0.902)	(0.841)
K_{mb} (mM)	0.177±0.024	0.326±0.072	0.175±0.062	0.562±0.134	0.647±0.170	0.401±0.264
		(0.504)	(0.348)		(0.659)	(0.613)
k_D (diffusion constant)		-	8515±4089		-	10353±10718
			(7801)			(2270)

Subscripts a: lower rate/higher affinity transporter (y^+L); b: higher rate/lower affinity transporter (y^+);

[1]preferred model in ECV_{304} (F=0.641 (n=180), p=0.42); note the high SEM for k_D; [2]preferred model in HUVEC (F=0.857 (n=23), p=0.37); note the high SEM for k_D

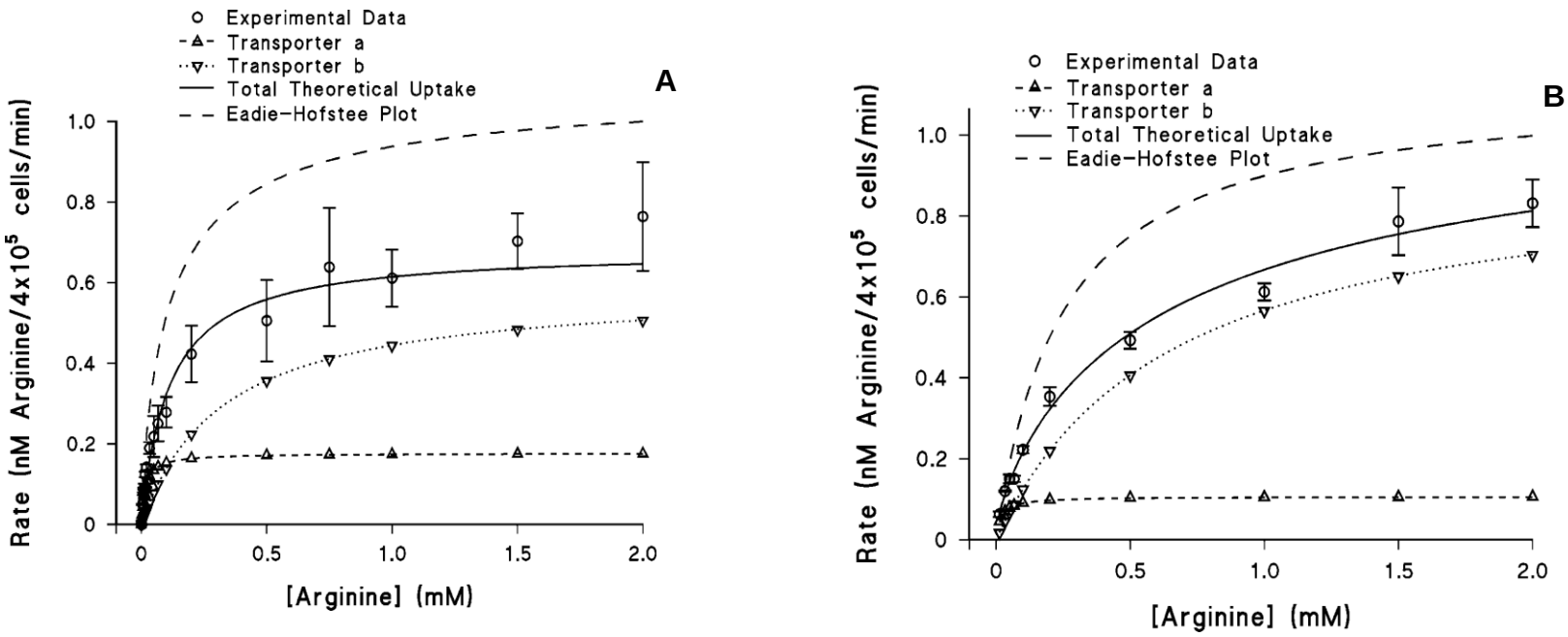


Figure 3.6 Data for ECV_{304} (Figure 3.6A) and HUVEC (Figure 3.6B) to determine kinetic constants. Mean±SD are from ten experiments (for ECV_{304}) and from duplicate experiments (for HUVEC). Michaelis-Menten graphs of dilution corrected experimental rate of arginine uptake (as mean±SD) and theoretical contributions of transporters '*a*' (y^+L; ….) and '*b*' (y^+; - - - -) and the combined total theoretical uptake (——) (derived from the constants obtained by non-linear modelling, Table 3.1), which agreed well with the experimental data. Also shown is the curve (– – –) calculated from the constants derived from the Eadie-Hofstee plots of dilution corrected data (Table 3. 1), which overestimated the experimental uptake. Experimental details have been described in sections 3.2.1 and 3.2.2, with uptake measured for 30 seconds.

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closely matched the experimental data (Table 3.1 and Figure 3.6A for ECV_{304} and Figure 3.6B for HUVEC respectively). For both cell lines, the y^+L transporter ('*a*') was the major contributor of uptake at low arginine concentrations and approached V_{maxa} above 0.300mM with near zero order kinetics above this concentration. The y^+ transporter ('*b*') accounted for most of the transport above this concentration.

Finally we re-calculated the kinetic constants using dilution corrected data, modelling an additive Michaelis-Menten equation to best fit the experimental data, to compare the results obtained with Eq. 2 of the non-linear modelling using the raw data as described above. From the Michaelis-Menten graph (Figure 3.6A) the constants determined for ECV_{304} cells were V_{maxa}=0.176±0.027nM arginine/4x10^5 cells/minute; K_{ma}=0.016±0.002mM; V_{maxb}=0.589±0.028nM arginine/4x10^5 cells/minute; and K_{mb}=0.326±0.072mM which were similar to those obtained from the non-linear modelling (i.e. the kinetic constants obtained from the model fit of Figure 3.6A matched those of the fit of Figure 3.2A; Table 3.1). Similar equivalent results were obtained for HUVEC: V_{maxa}=0.103±0.012nM arginine/4x10^5 cells/minute; K_{ma}=0.017±0.002mM; V_{maxb}=0.907±0.034nM arginine/4x10^5 cells/minute; K_{mb}=0.612 ±0.073mM (i.e. the kinetic constants obtained from the model fit of Figure 3.6B matched those of the fit of Figure 3.2A; Table 3.1).

3.3.3 Inhibition of arginine uptake by leucine

Leucine decreased both V_{maxa}' and K_{ma}' of tracer uptake by the high affinity/low rate transporter in ECV_{304}, at all the concentrations of leucine tested (Figure 3.7). V_{maxa}' decreased K_{ia}=0.024±0.003 and 0.159mM when leucine was <0.500mM and, equal to 0.500mM respectively.

The maximum rate of uptake (V_{maxb}') of the low affinity/high rate transporter in ECV_{304} was unchanged in the presence of leucine, except at 0.500mM leucine when V_{maxb}' was significantly lower ($p<0.0001$ for all leucine concentrations *vs* 0.500mM, Figure 3.7A). Compared to the uninhibited reaction, K_{mb}' was significantly decreased at leucine

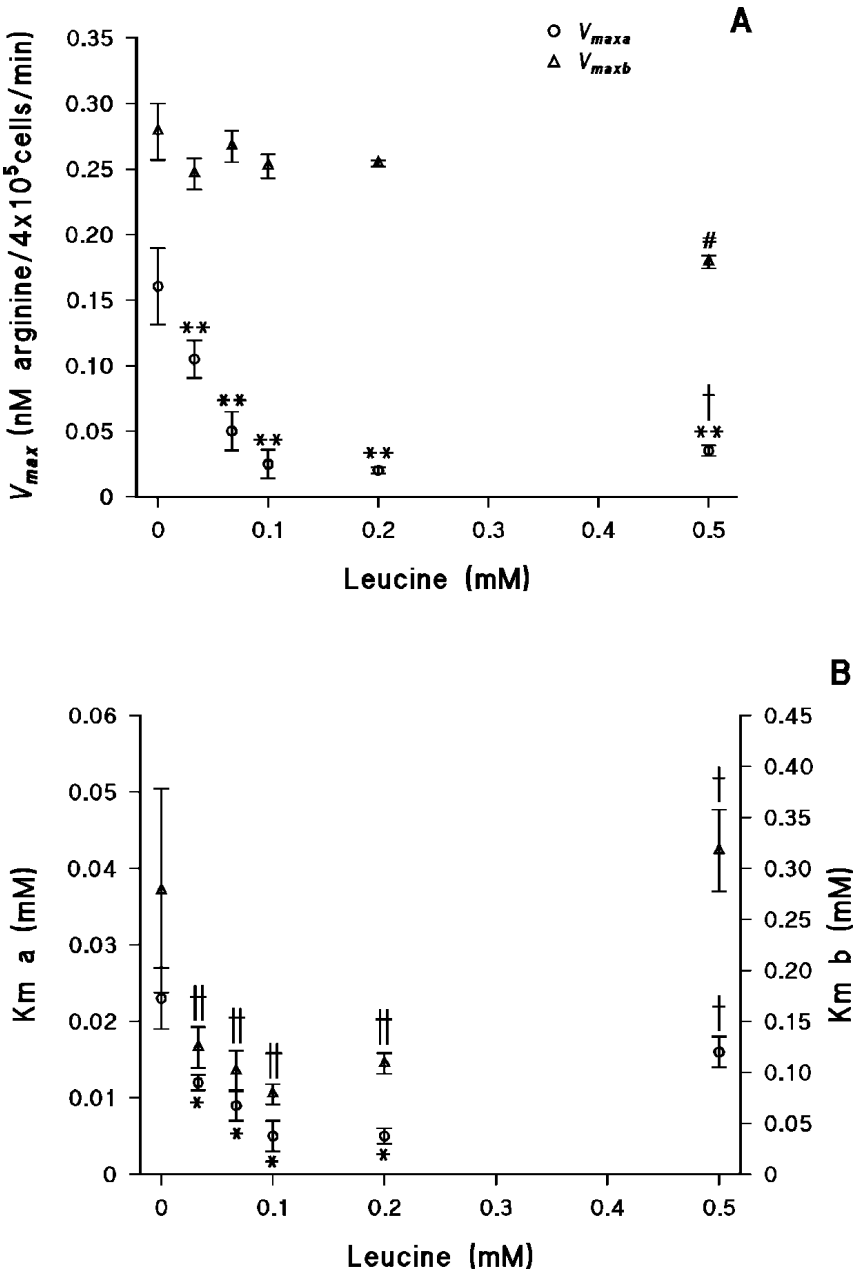

Figure 3.7 The effect of leucine on: A. The rate (V_{max}') of 10nM L-[^{3}H]arginine uptake and B. Michaelis-Menten constant (K_m') for the individual transporters, as determined by non-linear modelling. Results are mean±SD from triplicate experiments. The PBS tests solution contained 10nM labelled; various concentrations of unlabelled arginine with the concentrations of leucine as indicated in the graphs. *p<0.005 and **p<0.0005 *vs* no leucine; †p<0.005 *vs* 0.2mM leucine; #p<0.00001 *vs* all other leucine concentrations; ††p<0.05 *vs* no leucine.

concentrations between 0.033–0.2mM ($p<0.005$) but was not different to the uninhibited reaction at 0.500mM leucine ($p=0.35$; Figure 3.7B). The data suggests that such inhibition/activation was competitive for leucine concentrations between 0.033–0.2mM and was consistent with a model of "competitive activation" (Krupyanko; 2007) with K_{ib}=0.0.040±0.005 and K_{ib}=1.554mM when leucine was <0.500mM and 0.500mM respectively. There was insufficient data to determine whether V_{maxb} simply decreased linearly with increasing leucine which would suggest uncompetitive inhibition (unassociative) (Krupyanko; 2007).

The non-linear modelling suggested leucine affected both transporters differently without either transporter being completely inhibited at any of the concentrations of leucine tested. These kinetic constants were not determined at higher leucine concentrations.

3.3.4 Effect of NEM on arginine uptake in ECV_{304} cells

N-ethylmaleimide (0.2mM) incubated with the ECV_{304} cells for 10 minutes prior to the uptake of labelled arginine completely inhibited the low affinity/higher rate y^+ transporter. The result was tested using a single model of transport, which was preferred to a 2-transporter model of uptake, with the results for the latter model being ambiguous and both K_{mb}' and V_{maxb}' defaulting to a large value (∞, infinity) when using GraphPad Prism (Table 3.2) suggesting non-competitive inhibition.

3.3.5 Effect of BCH on arginine uptake in ECV_{304} cells

2-Aminobicyclo[2,2,1]heptanes-2-carboxylic acid (30mM) was pre-incubated with the ECV_{304} cells for 60 minutes prior to determining the tracer uptake. A two transport model was preferred over a single transport system ($p<0.0001$). V_{maxa}' and K_{ma}' were both significantly decreased ($p<0.0001$) whereas V_{maxb}' and K_{mb}' were unchanged (Table 3.2) suggesting that only the y^+L transport was affected by BCH.

Table 3.2 Effect of pre-incubation of the inhibitors NEM (0.2mM) and BCH (30mM) on arginine uptake by ECV_{304} cells. Results are mean±SEM from triplicate experiments and the robust values (less sensitive to outlier data) in brackets.

Difference between uptake with *vs* without inhibitor: *** $p<0.0001$.

	NEM		**BCH**	
	0mM	0.2mM	0mM	30mM
V_{maxa} (nM/4x10^5 cells/min)	0.166±0.0265 (0.224)	0.371±0.011 (-)	0.294±0.029 (0.362)	0.012±0.004 *** (0.010)
K_{ma} (mM)	0.024±0.003 (0.032)	0.059±0.003 (-)	0.026±0.002 (0.030)	0.002±0.001 *** (0.002)
V_{maxb} (nM/4x10^5 cells/min)	0.268±0.026 (0.3)	~ 5.10x10^{14} ^{***,1} (-)	1.509±0.071 (1.867)	2.109±0.102 (2.084)
K_{mb} (mM)	0.272±0.083 (0.762)	~ 2.08x10^{15} ^{***,1} (-)	0.598±0.085 (1.024)	0.602±0.049 (0.595)

[1] after pre-incubation with NEM, the best fit of V_{maxb} and K_{mb} defaulted to a large value (∞), with an ambiguous result. Hence, a single transport model with diffusion was preferred.

3.3.6 Sodium dependence of arginine uptake

Decreasing sodium from 157mM to 26mM affected neither V_{maxa}' nor K_{ma}', whereas the absence of sodium decreased both constants (Table 3.3 0mM *vs* 157mM: V_{maxa} $p<0.001$ and K_{ma} $p<0.01$; 0mM *vs* 26mM: V_{maxa} $p<0.01$ and K_{ma} $p<0.05$). K_{ma}'/V_{maxa}' was unchanged suggesting that the absence of sodium affected transport uncompetitively. V_{maxb}' of the low affinity/high rate transporter was significantly reduced when sodium was decreased or absent ($p<0.0005$; 0mM *vs* 26mM: p=ns) whereas, K_{mb}' was unchanged suggesting non-competitive inhibition of y^+ transport when sodium was decreased.

3.4 Discussion

In the present studies I used raw, rather than transformed data, in non-linear regression analysis to characterize the kinetics of arginine uptake into cells by more than one transporter, thereby avoiding the potential difficulties and errors of methods deriving constants from the linearization of the uptake process following Michaelian kinetics (Malo & Berteloot; 1991). Importantly, the modelling takes into account the concentrations of both the labelled and unlabelled arginine to be included and by determining changes in V_{max}' or K_m' was able to determine the inhibition type for either transporter in the presence of effector molecules.

Although I did not attempt to characterize all aspects of arginine uptake in the ECV_{304} cell line and in HUVEC, the values for the kinetic constants obtained, using this non-linear modelling approach, were in keeping with those reported for cationic amino acids in the literature (Devés & Boyd; 1998; Mann *et al*., 2003). The results show a) the effect of the unlabelled amino acid on the rate of uptake of the "trace" labelled amino acid was not linear and depended on the relative concentrations of unlabelled to labelled amino acid; b) the theoretical plot of the total rate of uptake, derived from the kinetic constants of arginine uptake estimated from Eadie-Hofstee plots significantly overestimated the experimental data; whereas c) when the constants were estimated from the non-linear modelling (Malo & Bertloot; 1991) there was good agreement. The results suggest that values of kinetic constants calculated from linearized data reported for cationic amino acids in the literature (Devés & Boyd; 1998; Mann *et al*., 2003)

Table 3.3 Kinetic constants for ECV_{304} cells determined at various sodium concentrations. Results are mean±SEM from triplicate experiments, the robust value (less sensitive to outlier data) in ( ) brackets and 95% C.I. for the slope in [ ] brackets. Significant differences: *p<0.05;**p<0.005;***p<0.0005:

0 or 26mM *vs* 157mM sodium; †p<0.005; ††p<0.0005: 0mM *vs* 26mM sodium.

	Sodium 157mM	**Sodium 26mM**	**Sodium 0mM**
V_{maxa}	0.142±0.027	0.201±0.036	0.1±0.007**†
(nM arginine/4x10^5 cells/min)	(0.184)	(0.236)	(0.118)
K_{ma} (mM)	0.016±0.002	0.018±0.003	0.011±0.001*††
	(0.019)	(0.021)	(0.012)
K_{ma}/V_{maxa}	0.112±0.005	0.09±0.004	0.107±0.002
	[0.098-0.12][1]	[0.083-0.1][1]	[0.103-0.111][1]
V_{maxb}	0.660±0.022	0.313±0.028***	0.349±0.009***
(nM arginine/4x10^5 cells/min)	(0.695)	(0.356)	(0.403)
K_{mb} (mM)	0.193±0.027	0.210±0.073	0.204±0.020
	(0.283)	(0.400)	(0.315)
K_{ma}/V_{maxa}	0.292±0.00	0.671±0.00	0.584±0.00
	[0.0-0.][1]	[0.0-0.][1]	[0.-0.][1]

may have overestimated the true values. Although Malo and Berteloot (1991) developed this approach for glucose uptake by more than one transporter, as far as I am aware this is the first report to show the discrepancy between theoretical and experimental data for amino acid uptake by more than one transporter.

Non-linear regression analysis allowed comparison between models of one or two transporters, the presence of diffusion (Malo & Berteloot; 1991) and the possibility of uptake by a single transporter with multiple binding sites (Chenu & Berteloot; 1993). The results consistently determined that, in the absence of inhibitors, a model of uptake by two independent transporters was preferred over other models. This conclusion was supported by curvilinear Eadie-Hofstee and Lineweaver-Burk plots using transformed data.

Studies have suggested that the uptake in HUVEC was facilitated by a single high-affinity transporter with a diffusion component, rather than two simultaneously operating transporters (Arancibia-Garavilla *et al*., 2003; Speake *et al.*, 2003). However, expression data suggests additional cationic transporters were operational in these cells (Rotmann *et al.*, 2007). The activity thus ascribed to the y^+L transporter could in fact be ascribed to more than one such transporter (Arancibia-Garavilla *et al*., 2003).

Differences between results of various studies may be partly explained by variations in study methodology, as studies have determined uptake in the presence of labelled amino acid alone (Sobrevia *et al*., 1995; Rotoli *et al*., 2005; Brunini *et al*., 2006), specified the activity, but not the concentration of trace labelled amino acid (Closs, Albritton *et al*., 1993; Casanello & Sobrevia; 2002; Hardy & May; 2002; Arancibia-Garavilla *et al*., 2003; Signorello *et al*., 2003; Martín *et al*., 2006; Rotmann *et al*., 2007) and other studies have reported results from studies using between 0.1-0.2μM (Ayuk *et al*., 2002; Schlaich *et al*., 2004), to 50μM labelled arginine (Durante *et al*., 1996) in the presence of fixed or varying concentrations of unlabelled substrate or inhibitors. My data showing non-linear uptake of tracer in the presence of unlabelled amino acid, underscores the importance of including the concentrations of both the labelled and unlabelled amino acid when determining the rate of uptake. The uptake of 10nM labelled amino acid in the presence of 0.025 and 0.100mM unlabelled amino acid

concentrations was not linear with time (Figure 3.1A), nor concentration [Figure 3.2A (ECV_{304}) and Figure 3.3A (HUVEC)]. These data underscore the importance ensuring linearity of uptake and including the concentrations of both the labelled and unlabelled amino acids when determining the rate of uptake.

Uptake by diffusion has been demonstrated as the non-saturable uptake of labelled arginine (0.05mM), measured in the presence of excess unlabelled arginine (10mM) (Speake *et al*., 2003; Martín *et al*., 2006). Subtracting this non-saturable uptake from the total transport (Dall'Asta *et al*., 2000; Arancibia-Garavilla *et al.*, 2003) may exceed the contribution of the high affinity/low rate transporter (y^+L) (Dall'Asta *et al*., 2000) which may be among the reasons that this transporter has been overlooked (Devés *et al.*, 1992). Subtracting the diffusion component from transformed data, results in the loss of statistical information, whereas when using the non-linear approach, such information is retained allowing error in the calculated kinetic constants to be determined (Malo & Berteloot; 1991). In my experiments using 10nM labelled arginine, the inclusion of the diffusion term did not improve the fit of the models and the contribution of diffusion to the total counts was insignificant. This does not suggest that the diffusion or the contribution of a non-saturable component is not important, but rather that under the experimental conditions used, this contribution was small.

3.4.1 Inhibitors and effector molecules

The non-linear approach makes no assumptions regarding the type of inhibition or activation of transport by effector molecules. Therefore, I investigated the inhibition of arginine uptake by leucine, which has been used to differentiate between y^+L and y^+ transport, NEM which inhibits y^+ transport, and the neutral amino acid analogue BCH, an inhibitor of sodium - independent transport (White, Gazzola *et al.*, 1982). Furthermore, I determined the effects of sodium on arginine uptake.

3.4.2 Leucine

Studies have used fitted models of competitive inhibition of one or both transporters to experimental data when determining the effects of neutral amino acid on cationic amino acid uptake (Devés *et al.*, 1992; Devés & Boyd, 1998; Hardy & May; 2002, Rotoli *et al.*, 2005). As the side chain of leucine and other neutral amino acids differs from that of arginine and lysine, it appears that models which included non-competitive and uncompetitive inhibition were not tested. Indeed, data showing K_i coinciding with I_{50} (50% inhibition of uptake) (Angelo *et al*., 1996), would be consistent with non-competitive or uncompetitive inhibition of the transport of leucine (Cheng & Prusoff; 1973).

My data showed that leucine concentrations of up to 0.200mM inhibited both transporters differently. Leucine affected the y^+L transporter by mixed inhibition ("discoordinated inhibition") and affected the y^+ transporter by "competitive activation" (Krupyanko; 2007). The results are in contrast to previous studies which assumed competitive inhibition of y^+L transport in erythrocytes (Devés *et al*., 1992), HUVEC (Arancibia-Garavilla *et al*., 2003) and platelets (Signorello *et al*., 2003). However, these reports did not determine inhibition by leucine at low and at physiological concentrations.

Although leucine did not completely inhibit transport at any of the concentrations tested, other studies, using higher leucine concentrations (1-10mM leucine in the presence of sodium), determined uptake of leucine-sensitive transport (i.e. the contribution of y^+L transport) by subtracting leucine-insensitive uptake (i.e. the contribution by y^+) from the total uptake (Mendes-Ribeiro *et al*., 1999; Arancibia-Garavilla *et al*., 2003; Martín *et al*., 2006; Rotmann *et al*., 2007). However, as stressed above, the results of such studies would depend on the relative concentrations of the added labelled tracer versus the unlabelled arginine present and were included in the models used in my study.

Different neutral amino acids affect arginine uptake differently (Closs, Albritton *et al*., 1993; Bröer *et al*., 2000; Rotmann *et al.*, 2007) and results may not be consistent if, for example, leucine was replaced by glutamine (Ayuk *et al*., 2002; Speake *et al*., 2003). In addition, there

appears to be no data suggesting that leucine and glutamine inhibit cationic transport in the same manner and such studies could be tested using this non-linear modelling approach.

3.4.3 NEM and BCH

The non-linear modelling approach determined that the y^+L transporter was unaffected by NEM, whereas the y^+ transporter was completely inhibited, which was consistent with the literature (Devés & Boyd; 1998; Babu *et al.*, 2003). Almost three decades ago, BCH was developed as model substrate for the sodium-independent system for neutral amino acids (White & Christensen; 1982b). Interestingly, the results of the present study show that BCH significantly reduced the activity of the y^+L transporter without affecting the y^+ transporter. In the absence of a y^+L transporter inhibitor, BCH or structurally related analogues may be of interest in the quest to obtain a selective inhibitor for this transporter.

3.4.4 Sodium dependence of arginine transport

Cationic y^+L and y^+ amino acid transport is accepted as being independent of sodium concentration (Devés & Boyd; 1998; Mann *et al.*, 2003; Bröer; 2008). White originally noted inhibition of cationic amino acid transport by neutral amino acids, such as leucine, glutamine, methionine, and to a lesser extent, homoserine and serine (White & Christensen; 1982b; Devés *et al*., 1992). Such sodium-dependent inhibition of cationic transporters was used to identify the y^+L transporter (Devés *et al*., 1992) and has been used to differentiate this transporter from the y^+ transporter (Mendes-Ribeiro *et al*., 1999; Rojas & Devés; 1999; Arancibia-Garavilla *et al*., 2003; Martín *et al*., 2006; Rotmann *et al*., 2007). However, data suggests that neutral amino acids also inhibit uptake of cationic amino acids by y^+ transport (Christensen; 1990) with some 30% of lysine uptake into HUVEC (Hardy & May; 2002) and BAEC (Durante *et al*., 1996) being sodium-dependent. The latter could be explained if other transporters were operational in these cells, but such discrepancies in the literature highlight the difficulties in comparing cationic transport studies and appear to be similar to those noted for epithelial cells (reviewed by Bröer; 2008).

Although it was suggested that sodium occupies the site normally occupied by the ω-amino group of cationic amino acids to facilitate neutral amino acid transport (Christensen & Antonioli; 1969), if sodium was already occupying the ω-amino group site, it is not clear how these transporters facilitate cationic amino acid uptake. My results may suggest that sodium ions are necessary for the conformational integrity of the transporters or were required for transport of arginine, i.e. sodium interacts with the arginine-transporter complex.

Three concentrations of sodium were used to determine the effects of sodium on arginine uptake in ECV_{304} cells. In contrast to earlier findings, the results suggest that y^+L transport was relatively unaffected by sodium concentration unless sodium was absent when transport was uncompetitively inhibited. In contrast, the rate of arginine uptake by the y^+ transporter was significantly reduced as sodium was decreased, without affecting the affinity of this transporter (i.e. non-competitively inhibited). The results suggest that the absence of sodium would decrease the total rate of uptake by both transporters but would only affect the affinity of the y^+L transporter. These data underscore the advantage of measuring uptake over a wide range, not single concentrations of unlabelled substrate, to determine the kinetic constants of both transporters, which is possible using this non-linear approach.

3.5 Study limitations

Although the origin of ECV_{304} cells is controversial (Brown *et al*., 2000; Drexler *et al*., 2002, Mann *et al*., 2003), these cells express hCAT1 (y^+) and y^+LAT2 (y^+L) transporters (Rotmann *et al*., 2007), and were therefore used as a model to determine uptake by more than one transporter.

We depleted cells of arginine for 24 hours (Sessa *et al*., 1990) with other studies using times between 1 and 24 hours (Mann *et al*., 2003). Intracellular arginine levels appear to be maintained in HUVEC over this time (Mann *et al*., 2003), and we did not determine whether shorter depletion periods affected the rate of arginine uptake. The initial rate of arginine uptake was then measured for 30 seconds assuming that both the conversion of labelled arginine to metabolites, which may be exported, and the export of arginine via trans-

stimulation (Flores *et al.*, 2003), was insignificant. Furthermore, our results were in agreement with the literature (Mann *et al*., 2003).

Although, HUVEC also express other transporters, hCAT1, -2B, y+LAT1 and 2 (Rotmann *et al*., 2007) and under the experimental conditions, the study showed statistically that a two transport model was preferred over those of a single or three (6 unknowns) transporter model, with or without multiple binding sites. The possibility of a single transporter modified by the binding of a regulatory subunit, or by forming a dimeric transporter (Oulianova & Berteloot; 1996) may explain many of the findings, and would be the subject of future investigations.

The effects of sodium on uptake of arginine in the presence of leucine, particularly at 1-10mM leucine concentrations, and other neutral amino acids still remain to be determined by this method. In the absence of data measuring labelled arginine uptake in the presence of mM leucine concentrations, at this time we have no explanation for the observation of a change in the rate of arginine uptake at 0.5mM leucine. As V_{maxb} appeared to decrease with increasing leucine concentrations, the inhibition may simply be uncompetitive inhibition (unassociative; Krupyanko; 2007) but there were insufficient data to substantiate this possibility. Moreover, the possibility that leucine and sodium affects the membrane potential (or pH in the case of sodium), thereby influencing arginine transport, was not investigated in our study and cannot be excluded (Devés & Boyd; 1998).

3.6 Conclusions

This non-linear modelling approach that I have used to characterise the uptake of arginine in cells using raw, rather than transformed data, avoids the difficulties and errors of methods deriving constants from the linearization of uptake processes following Michaelian kinetics. Moreover, the model allows for more than one transporter to be modelled, accounts for the concentrations of both labelled and unlabelled arginine, and allows the constants to be determined over a range of unlabelled, not single, substrate or inhibitor concentrations, without making assumptions regarding the type of inhibition/activation present. The theoretical models of total uptake closely matched the experimental data, uptake curves were

obtained for the individual transporters, and effects of inhibitors and sodium on the individual transporters determined. Finally, my study underscores the value of using a non-linear regression modelling approach, which more accurately represents the kinetics of cationic amino acid uptake, activation and inhibition into cells and hence provides explanations for discrepancies in the literature. More importantly, in conjunction with these kinetic studies, the effects of limited arginine and increased homocystine concentrations on NO production can be determined.

CHAPTER 4

The effect of homocystine on arginine uptake and nitric oxide production

4.0 Abstract

Studies have shown an elevation in routinely measured plasma tHcy concentrations to be an independent risk factor for cardiovascular disease. Large prospective vitamin supplementation trials conducted in patients with existing cardiovascular disease showed a decrease in plasma tHcy. However, decreased plasma tHcy showed no subsequent reduction in cardiovascular events. As only a fraction of tHcy is actually present as the free reduced sulphydryl which is responsible for vascular effects, tHcy may be simply a marker of cardiovascular risk. However, the disulphide, homocystine, although almost undetectable in plasma of subjects without disease, has been determined to be 1-2μM in chronic renal failure patients, and as high as 7μM in homocystinuric children. As homocystine shares common transport with the NO precursor, arginine, we determined whether homocystine decreased arginine uptake into cells and/or affected NO production.

Uptake of labelled L-[^{3}H]arginine was measured in confluent, arginine depleted HUVEC and ECV_{304} cells with unlabelled arginine, without or with homocystine and modifiers. 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.

Concentrations of 2.5μM homocystine, observed in patients with severe renal disease, inhibited arginine uptake by 90% by $y^{+}L$ transport in both HUVEC ($p<0.0005$) and in ECV_{304} cells ($p<0.05$) and reduced the K_{ma} of $y^{+}L$ transport in HUVEC ($p<0.0001$) and in ECV_{304} cells ($p<0.002$), affecting uptake in a competitive-like manner. Inhibition of arginine uptake following 24 hours incubation of ECV_{304} cells with homocystine could be reversed by arginine. In contrast, homocystine increased arginine uptake by y^{+} transport in HUVEC

($p<0.01$) but not in ECV_{304} cells. However, effects of homocystine on the rate of NO production could not be shown in the experimental model.

By demonstrating that homocystine significantly inhibited arginine uptake by y^+L transport in both HUVEC and ECV_{304} cells, these data provide a mechanism by which homocystine may affect intracellular arginine concentrations especially when extracellular arginine concentrations are low.

4.1 Introduction

Retrospective and case-control studies have shown elevated plasma tHcy concentrations to be an independent risk factor and predictor of vascular (Boushey *et al.,* 1995) and cardiovascular disease (Christen *et al.,* 2000). A meta-analysis of prospective studies has shown a 16% increase in the hazard ratio for recurrent cardiovascular events (Wald *et al.,* 2002) with a 5µM increase in tHcy. Clinical trials addressing supplementation with vitamin cofactors, folic acid, vitamin B6 and vitamin B12, involved in the metabolism and re-methylation of homocysteine, have demonstrated significant reductions in plasma tHcy concentrations and improvement in vascular function (Doshi *et al.,* 2002; Moat *et al.,* 2003). Consequently, to determine the benefit of such intervention on recurring cardiovascular events, large prospective supplementation trials were conducted in patients with existing cardiovascular disease. Although demonstrating significant reductions in plasma tHcy concentrations, none of these trials (Wierzbicki; 2007) showed reductions in vascular events (Toole *et al.,* 2004), recurrence of myocardial infarction, stroke or sudden death due to coronary artery disease (Bønaa *et al.,* 2006; The HOPE-2 Investigators, 2006). Thus, these results are inconsistent with the considerable data showing an association of elevated homocysteine with increased risk of cardiovascular disease (Boushey *et al.,* 1995).

Although plasma tHcy levels are routinely measured (Ubbink; 2000), only 1.5 - 4% of the tHcy is actually present as the free reduced sulphydryl (-SH or thiol; 0.1µM) form of homocysteine (rHcy). Free oxidized homocysteine, present as the homocysteine disulphide,

Table 4.1 Summary of the various concentrations of the forms of homocysteine measured in biological fluids.

	Total homocysteine (µM)	Free reduced homocysteine (µM)	Free oxidized homocysteine (µM)	Free homocystine (µM)	References
Control subjects (n=15)	11±3	0.09±0.03	3.6±1.4		Sjöberg *et al.*, 2006
Controls	7.8±0.3 (7.5)		2.3	(2.1±0.1?)*	Melnyk *et al.*, 1999
Controls	9.5±1.7	0.14±0.03	2.1±0.5		Andersson *et al.*, 1995
Cerebral infarct	15.7±5.0	0.19±0.07	3.5±1.0		
Homocystinuria	258	12.7	153		
Controls			3.4±0.2		Wilcken *et al.*, 1983
Controls			3.7±0.2		
Heterozygotes*			3.3±0.3		
Ischemic heart Disease			3.6±0.3		
IHD (twin, n=1)			8.1	1.2	
Controls (20)	6.5±0.3	0.18±0.01	1.8±0.1 (1.6±0.1)+		Williams *et al.*, 2001
Stroke (20)	9.9±0.6	0.27±0.02	2.8±0.2 (2.6±0.2)+		
Controls	10.3	0.18	2.2 (1.9)+		Yang *et al.*, 2004
Stroke	10.7	0.24	2.3 (2.0)+		
Heathy men (n=10)	9.7±2.0	0.17±0.05	2.4±0.6		
Controls (n=22)			3.1±1.1	0	Andersson *et al.*, 1993
Chronic renal failure (n=22)			8.2±3.4	1.7±0.6	
Homocystinuric Children (n=7)		9.1±7.2		7.2±2.2	Wilcken & Gupta; 1979
Homocystinuric Children (n=6)	10.12±7.33			7.23±2.17	Perry *et al.*, 1967

*Result differs substantially from all other studies. Electrochemical detection was used. + Free oxidized homocysteine

homocystine, and the mixed disulphide (with cysteine) accounts for 20 – 30% of tHcy, with the remaining 70 – 80% of the tHcy being protein bound (Mansoor *et al.*, 1992; Andersson *et al.*, 1993; 1995). The concentrations of the various forms of homocysteine measured in biological fluids are summarised in Table 4.1. As the atherogenic effects of homocysteine have been attributed to the reactive free sulphydryl, rather than the oxidized or protein bound forms, finding associations between tHcy and cardiovascular disease may not be appropriate (Stamm & Reynolds; 1999; Wagner & Koury; 2007). It has therefore been suggested that elevated plasma tHcy may simply be a marker, rather than a cause of cardiovascular disease (Loscalzo *et al.*, 2006). In the absence of a clear mechanism by which homocysteine causes vascular disease (Wagner & Koury; 2007), one of the other species making up the tHcy may be contributing to cardiovascular disease through a different mechanism which may not involve the free sulphydryl.

The common transport of cationic amino acids was first suggested when excessive urinary excretion of arginine, lysine and cystine was observed in cystinuric patients (Dent & Rose; 1951). This was demonstrated almost 20 years later (Cusworth & Gattereau; 1968), and by studies showing common transport of arginine, lysine and homocystine in isolated nephrons (Foreman *et al.*, 1980; Bovee & Segal; 1984), and subsequently from *in vitro* studies using various cell types including endothelial cells (Devés & Boyd; 1998; Mann *et al*., 2003; Bröer; 2008). Importantly, the earlier studies demonstrated that the homocysteine disulphide, homocystine, was taken up by this same common transport system in isolated nephrons and competed for uptake with arginine and lysine (Cusworth & Gattereau; 1968; Foreman *et al.*, 1982). These studies may suggest that homocystine, by reducing arginine uptake may impact on NO production in other cell types. Therefore, the aim of this study was to determine the effects of homocystine on cellular arginine uptake and hence on NO production.

I adapted the general non-linear approach of Malo and Berteloot; (1991) to model arginine uptake into both HUVEC and ECV_{304} cells (Nel *et al.*, 2012). The method used the initial rate of uptake to determine the kinetic constants for two or more amino acid transporters acting simultaneously in these cells. Such an approach, by including the concentrations of both the trace radioactive labelled and the unlabelled amino acid overcame the limitations and errors in

methods deriving constants from the linearization of the uptake processes following Michaelian kinetics (Malo & Bertloot; 1991). Furthermore, in the presence of an inhibitor, the type of inhibition could be determined from changes in the constants and as the uptake by each transporter was additive, the theoretical contribution of uptake by each transporter could be modelled, enabling the contribution of each transporter to be determined (Nel *et al.,* 2012).

4.2 Materials and methods

4.2.1 Cell culture

The ECV_{304} and HUVEC endothelial cells were cultured as described in sections 2.2.1 and 2.2.3 respectively, and illustrated in figure 2.1 of Chapter 2.

4.2.2 Arginine uptake methodology

Homocystine was purchased from Sigma-Aldrich, St Louis, Ms, USA and dissolved in PBS at various concentrations.

HUVEC and ECV_{304} cell cultures were used for the 30 second uptake assay, which was performed as described in section 2.3.1 and as illustrated in figure 2.2 of Chapter 2. However, to determine whether extended incubation of the cells with homocystine would affect the uptake of arginine, and whether arginine could overcome such effects, ECV_{304} cells were incubated with 0µM, 12.5µM and 25µM homocystine in the presence of 0.0 – 150µM arginine for 24 hours at 37°C, after the initial 24 hour arginine depletion period as described in section 2.3.1. The pre-incubation experiments were only done in ECV_{304} cells due to the short lifespan of the primary HUVEC cultures. For the extended incubations, the cells were washed twice with PBS after the 24 hour incubation with homocystine and arginine, after which the 30 second L-[^{3}H]arginine uptake assay was performed as described in section 2.3.1 and as illustrated in figure 2.2 of Chapter 2.

Arginine uptake kinetics were determined by using Eq's 1 and 2 as described in section 3.2.1 and the statistical analysis as described in section 3.2.2 for arginine uptake in the presence of one inhibitor of Chapter 3.

The methodology for including NEM in the assay was as described in the second paragraph of section 3.2 of Chapter 3.

4.2.3 Detection of nitric oxide

Although endothelial cells express NOS constitutively, due to their low levels of NOS protein expression they produce very small amounts of NOS. Hence, detection of such small amounts of NO with chemiluminescence, spectrophotometry, oxyhaemoglobin, fluorometry and voltammetric assays are not sensitive enough and none of these systems could previously be used for real time NO production detection in living cells (Nakatsubo *et al.,* 1998). The diaminofluoresceine dye, DAF, was developed and used with success to detect real time NO by fluorometry, in the supernatant of BAEC. The reaction of NO with DAF gives a bright green fluorescent triazolofluorescein detectable by a fluorometer. The BAEC grown to confluency in a 24 well cell culture plate, produced 300nM NO after a two hour incubation period (Nakatsubo *et al.,* 1998).

The mechanism of the reaction of DAF with NO involves N_2O_3 being generated as follows: $2NO + O_2 \rightarrow 2NO_2$ and $2NO_2 + 2NO \leftrightarrow 2N_2O_3$ (Nakatsubo *et al.,* 1998). When using MAHMA.NONOate (NOC-9) as a NO donor, 100nM NOC-9 generated 200nM NO while consuming 0.44nM DAF, indicating that DAF used at a concentration of 0.1μM is not rate limiting (Leikert *et al.,* 2001).

The detection of NO by DAF-2 has limitations in that the mechanism of the reaction between NO and DAF-2 is complex since DAF-2 does not react with NO itself but with an active intermediate, N_2O_3 which is the oxidized form of NO (Nakatsubo *et al.,* 1998). Therefore, the resulting fluorescence intensity does not only depend on the concentrations of NO and DAF-2, but also on the rate of NO oxidation (Leikert *et al.,* 2001). However, this method proved

sufficient to demonstrate the production of NO by rat cardiomyocytes (Strijdom *et al.*, 2004) and by rat cardiac microvessel endothelial cells (Strijdom *et al.*, 2005).

4.2.3.1 Nitric oxide detection methodology

All the solutions and cell washes for the NO production experiments with or without arginine, homocystine, drugs or other modifiers, consisted of Krebs Henseleit buffer (as previously described by Arancibia-Garavilla *et al.*, 2003) with Ca^{2+} instead of Na^{+} (see Appendice A), to ensure optimal results with the DAF-2 NO sensitive dye (as recommended by the manufacturer [(Sigma-Aldrich, Chemie, GmbH) in a personal e-mail communication].

Standard curves were established with the NO generator, DEA/NO as described in section 2.3.2.1, in order to ensure optimal NO specificity. The cellular NO detection assay was as described in section 2.3.2.2 and illustrated in figure 2.3 of Chapter 2.

4.3 Results

4.3.1 Preliminary studies

Preliminary studies established that L-[^{3}H]arginine uptake by the cells was linear up to 50nM arginine (Figure 3.1A of Chapter 3) and for up to 5 minutes (Figure 3.1B of Chapter 3). The initial rate of 10nM L-[^{3}H]arginine uptake was calculated over a 30 second time period which was used in all experiments. In the presence of unlabelled arginine, the rate of 10nM L-[^{3}H]arginine uptake was dependent on both the concentration of L-[^{3}H]arginine and unlabelled arginine and decreased in an exponential manner (Figure 3.1C of Chapter 3).

4.3.2 Modeling arginine uptake

A model of arginine uptake by both HUVEC and ECV_{304} cells of two transporters without a diffusion component was preferred over either a single model of uptake or a model of two transporters with a diffusion component (Nel *et al.*, 2012; Figure 4.1A). A model which

included a third transporter (6 unknowns, with an extra term) did not improve the model fit of the experimental data (data not shown). Therefore, in this study we modelled arginine uptake by two transporters without diffusion, and obtained values for the constants which were similar to that reported in the literature (Mann *et al*., 2003). The ratio of V_{maxa} to V_{maxb} was greater in HUVEC (V_{maxa}/V_{maxb} = 0.19) compared to in the ECV_{304} cells (V_{maxa}/V_{maxb} =0.10).

4.3.3 Effect of homocystine on arginine uptake

At all concentrations of homocystine, the model of two transporters without diffusion was preferred over a single transporter model for both HUVEC and ECV_{304}, indicating that neither transporter was completely inhibited at any of the concentrations of homocystine tested (Figure 4.1A). Furthermore, after correcting for dilution, the generated Eadie-Hofstee plots were curvilinear (as illustrated in HUVEC for a homocystine concentration of 12.5µM; Figure 4.1B), supporting the conclusion that two transporters were operating simultaneously.

The Michaelis-Menten curve of total arginine uptake by HUVEC (Figure 4.1C) showed that 12.5µM homocystine had little effect on total arginine uptake whereas 25µM homocystine appeared to enhance total arginine uptake. The model allowed the theoretical uptake by the individual transporters to be calculated, and as illustrated for 12.5µM homocystine (Figure 4.1D), the theoretical arginine uptake by y^+L transport (V_{maxa}) was decreased, whereas uptake by y^+ transport (V_{maxb}) appeared to be unaffected.

Figure 4.2 summarizes the effects of homocystine on V_{max} and K_m of each transporter facilitating arginine uptake for HUVEC (data for ECV_{304} cells has not been shown). In both HUVEC and ECV_{304} cells, homocystine inhibited arginine uptake by y^+L transport, significantly decreasing V_{maxa} at all concentrations of homocystine tested in HUVEC ($p<0.0005$; Figure 4.2A), whereas in ECV_{304} cells V_{maxa} was only decreased when homocystine concentrations were less than 12.5µM ($p<0.05$). Homocystine significantly reduced the K_{ma} of y^+L transport in HUVEC ($p<0.0001$; Figure 4.2B) at all concentrations tested and the

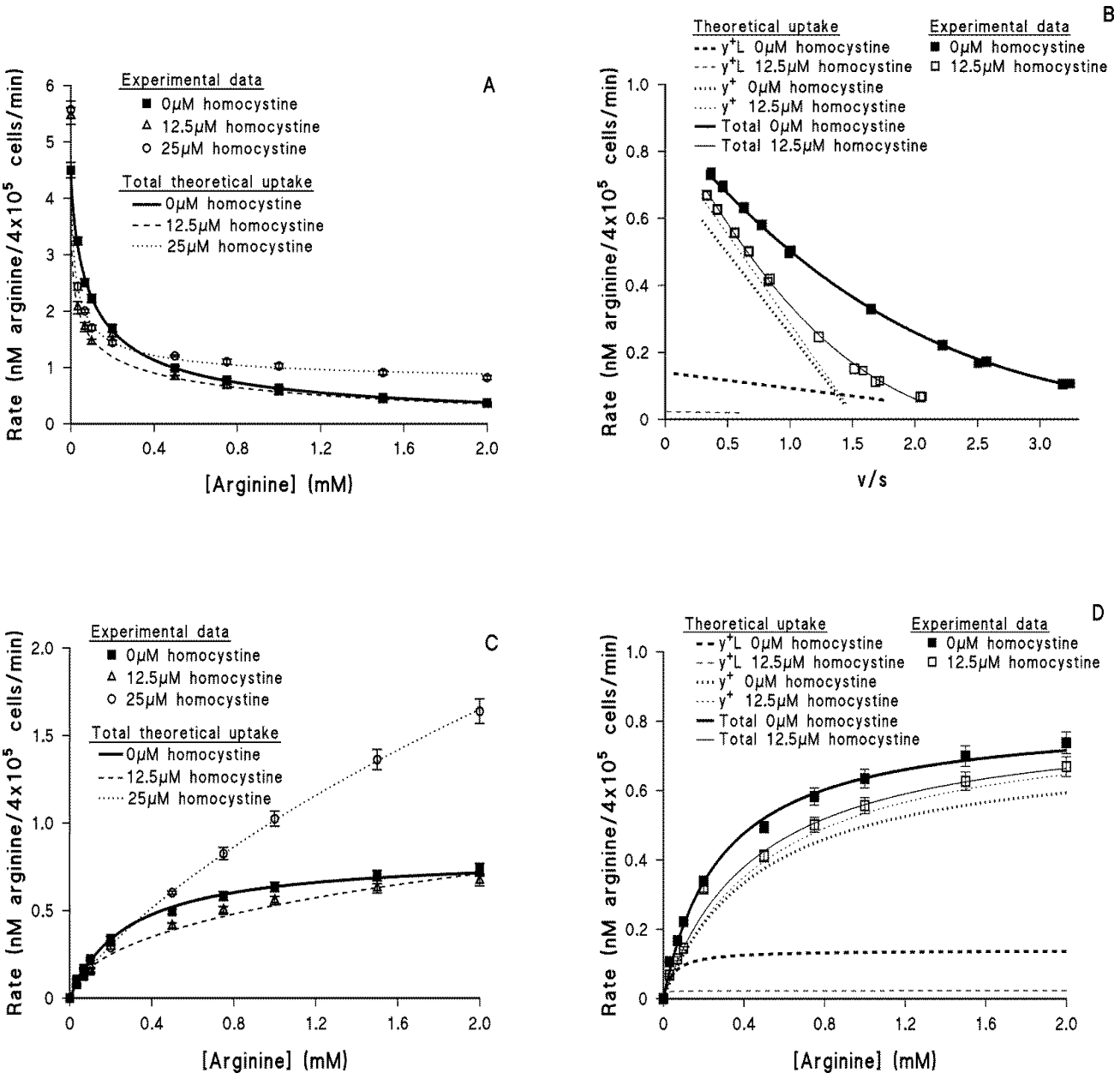

Figure 4.1 **A.** Uptake of L-[^{3}H]arginine by HUVEC in the presence of unlabelled arginine used to calculate the kinetic constants of uptake by non-linear modelling (Nel *et al.*, 2012), showing uninhibited uptake and illustrating the effect of 12.5μM and 25μM homocystine. Experimental and theoretical uptake has been shown. **B**. Eadie-Hofstee plots showing the two distinct components of uptake. Data for uninhibited uptake and uptake in the presence of 12.5μM homocystine is shown. The difference between the theoretical uptake by the individual transporters, derived from the calculated kinetic constants, and the total experimental uptake is shown. **C**. Michaelis-Menten plots of total uptake derived from dilution corrected data shown in A for homocystine concentrations of 12.5μM and 25μM showing agreement of the theoretical curve with experimentally derived data. **D**. Theoretical Michaelis-Menten plots of total uptake and that calculated for the individual transporters showing the inhibition of y^+L transport by 12.5μM homocystine (see Figure 4.3).

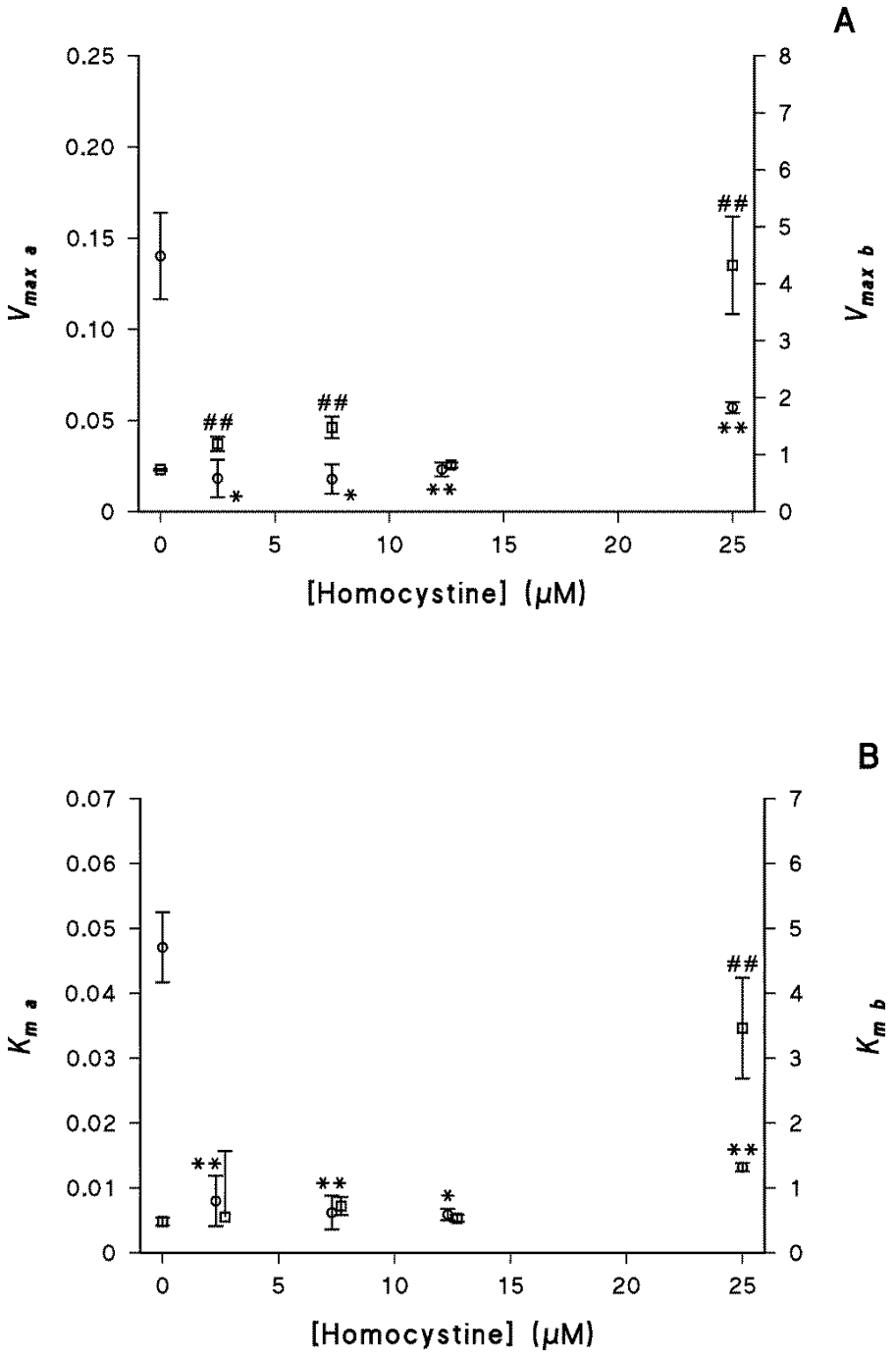

Figure 4.2 The effect of homocystine on the kinetic constants, **A.** V_{max}' and **B.** K_m', for the transporters facilitating arginine uptake in HUVEC. Data as mean±sem.
○ y^+L ('a') transporter; □ y^+ ('b') transporter.
Differences from homocystine = 0μM: * $p<0.05$; ** $p<0.005$ for y^+L ('a') transporter and ## $p<0.005$ for y^+ ('b') transporter.

K_{ma}'/V_{maxa}' was decreased when homocystine concentrations exceeded 12.5μM, although with the low values of both K_{ma}' and V_{maxa}', interpretation of these data is difficult. For ECV_{304} cells, K_{ma} was significantly decreased when homocystine concentrations were less than 25μM. This would be consistent with competitive, or associative activation (Krupyanko; 2007) of uptake by y^+L transport. Homocystine increased V_{maxb} ($p<0.01$) in HUVEC at all concentrations tested (Figure 4.2A), whereas in ECV_{304} V_{maxb} was only significantly increased when homocystine concentrations exceeded 18μM (data not shown). In both HUVEC (Figures 4.2B) and ECV_{304} cells (data not shown), K_{mb} of y^+ transport was unchanged below 25μM homocystine. This would suggest activation of y^+ transport by homocystine, in a non-competitive manner (Krupyanko; 2007).

The modelling approach allowed the theoretical Michaelis-Menten plots to be derived from the kinetic constants shown in Figure 4.1, for each individual transporter (Figure 4.3). Importantly, there was a 90% reduction in y^+L transport (V_{maxa}') at 2.5μM homocystine in both HUVEC and ECV_{304} cells (Figure 4.3A and 4.3B respectively), and in ECV_{304} cells, 5μM homocystine reduced V_{maxa}' by 97% inhibition (Figure 4.3B). Inhibition by homocystine could not be reversed even in the presence of high concentrations of exogenously added arginine in either cell type. In contrast, arginine uptake by y^+ transport was enhanced by homocystine in HUVEC but essentially unchanged in the ECV_{304} cells (Figure 4.3C and 4.3D respectively).

4.3.4 Effect of pre-incubation with both arginine and homocystine

In ECV_{304} cells, incubation with 0μM - 150μM arginine with either 12.5μM or 25μM homocystine, for 24 hours, did not change the kinetics of trace L-[^{3}H]arginine uptake by y^+L transport (Figure 4.4A). However, 12.5μM, but not 25μM homocystine, decreased the rate of arginine uptake by the y^+ transporter ($V_{maxb;}$ $p<0.005$; Figure 4.4B). The K_{ma} and K_{mb} were unchanged, suggesting homocystine did not affect the affinity of uptake for either transporter (Figures 4.4C & Figure 4.4D respectively).

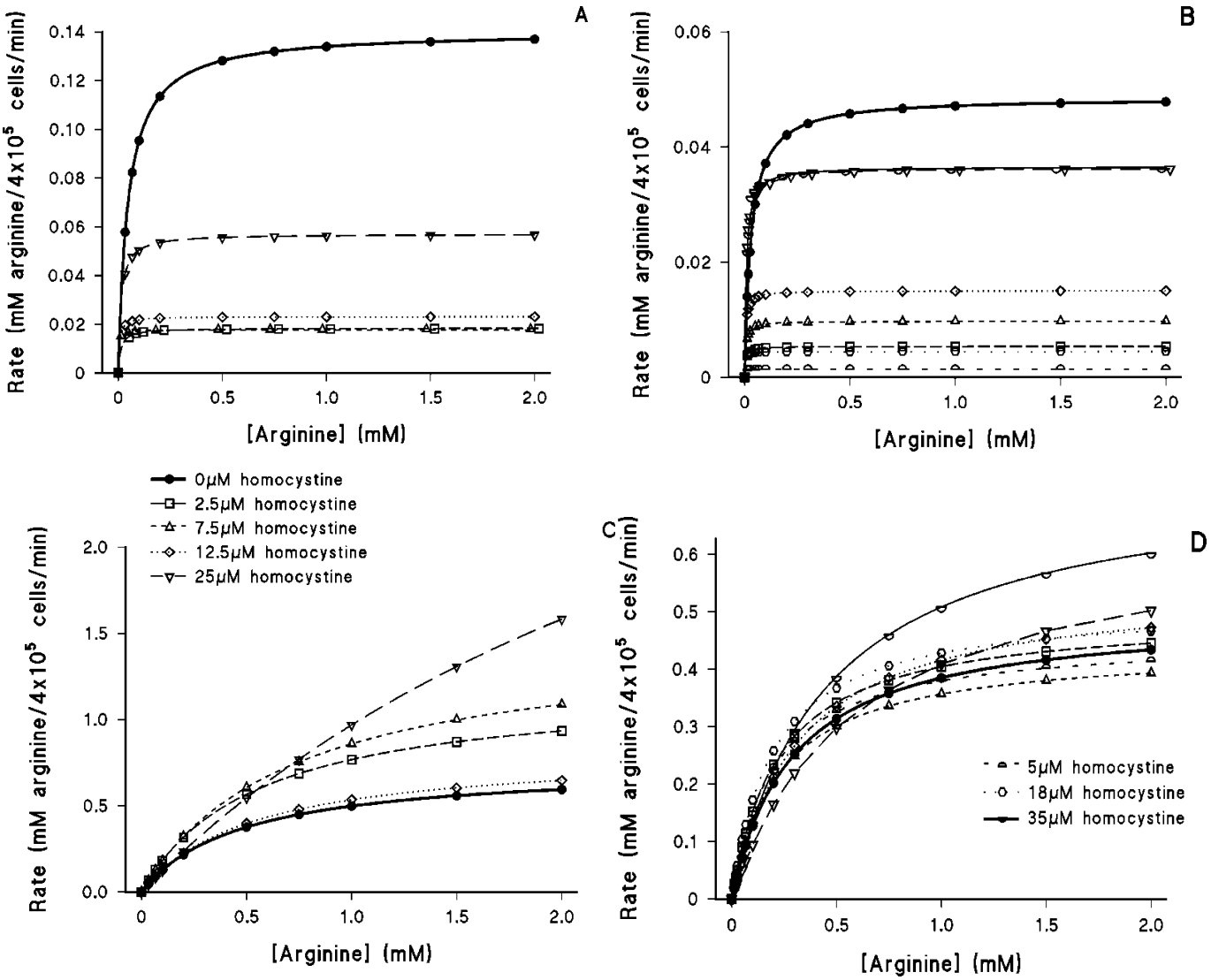

Figure 4.3 Michaelis-Menten plot of theoretical uptake by the individual transport in the presence of homocystine by y^+L uptake: **Upper panel**: A. HUVEC (left) and B. ECV_{304} cells (right) and by y^+ uptake: **Lower panel**: C. HUVEC (left) and D. ECV_{304} cells (right). The y^+L uptake was almost completely inhibited by homocystine whereas y^+ uptake was essentially unchanged for homocystine concentrations below 25μM.

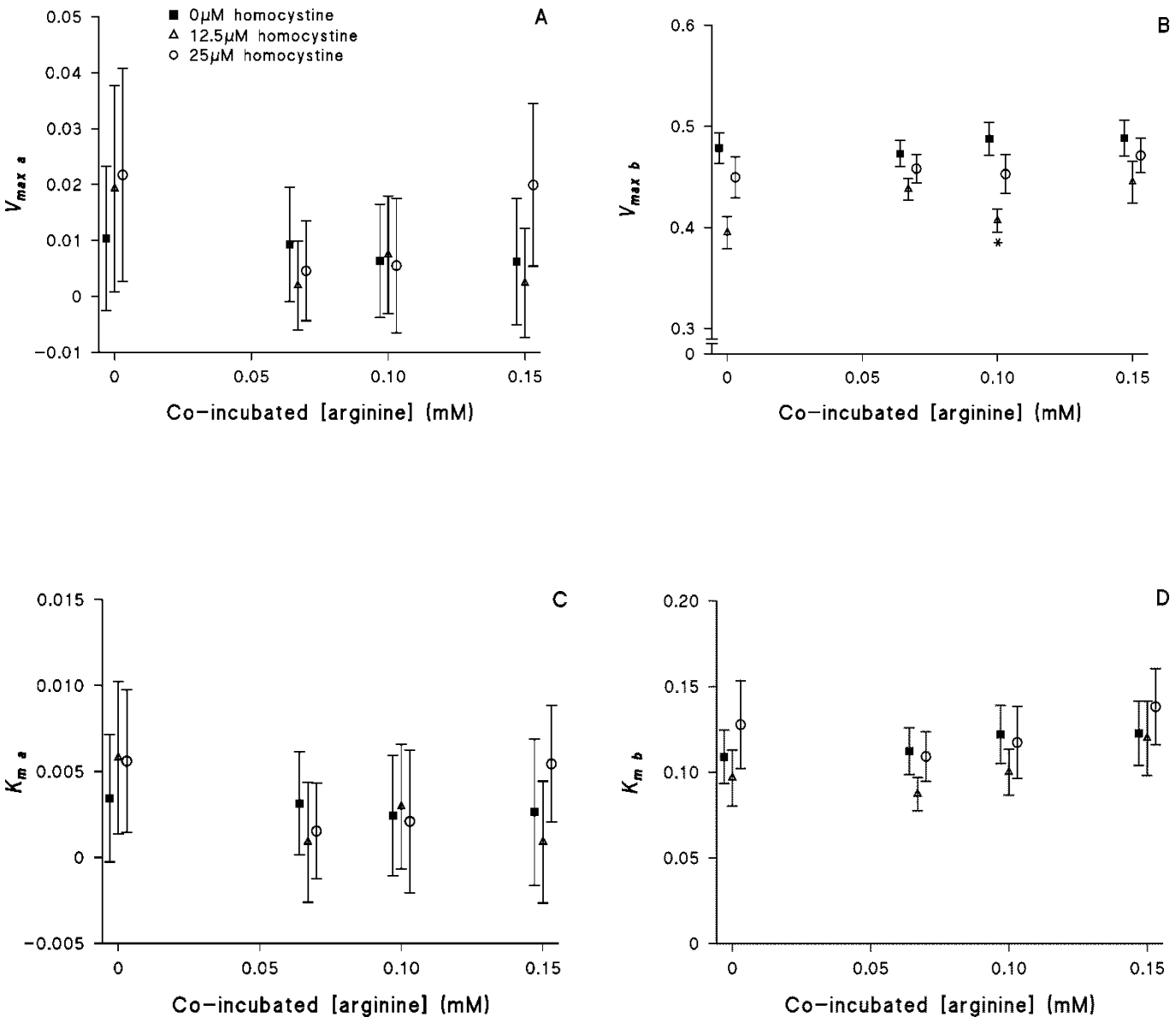

Figure 4.4 The effect of 24 hour co-incubation of homocystine with arginine on the kinetic constants of arginine uptake in ECV_{304} cells. 0µM, 12.5µM and 25µM homocystine were co-incubated with 0µM - 150µM arginine prior to determining L-[^{3}H]arginine (10nM) uptake (see experimental). **Upper panel**: A. V_{maxa} (left); B. V_{maxb} (right); **Lower panel: C.** K_{ma} (left); D. K_{mb} (right) for the transporters y^+L ('a') and y^+ ('b') respectively. *p<0.005 *vs* 0µM Homocystine.

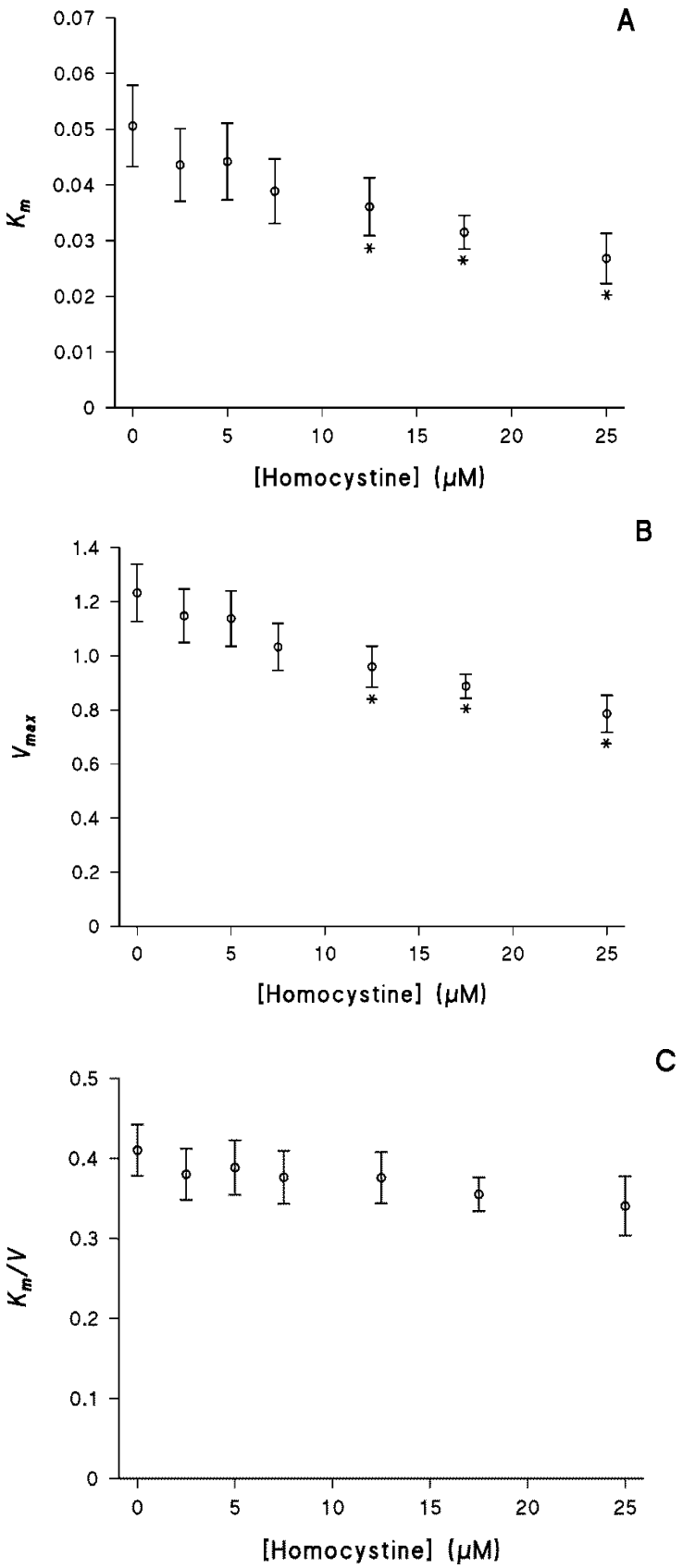

Figure 4.5 Effect of a 10 minute pre-incubation with NEM on arginine uptake in ECV_{304} cells, in the presence of varying homocystine concentrations on the V_{max}', K_m' and the ratio K_m'/V_{max}'. In the presence of NEM a model of uptake by a single transporter (y^+L) was preferred over uptake by two independent transporters. *$p<0.05$ *vs* 0μM Homocystine. The changes in the kinetic parameters are as indicated in the graphs.

4.3.5 Effect of *N*-ethylmaleimide on arginine uptake in the presence of homocystine

In order to compare the results of the present study to previous studies, we determined the effects of NEM on arginine uptake in the presence of homocystine. NEM selectively inhibited arginine uptake by y^+ transport (Devés & Boyd; 1998) and my results showed significant inhibition of y^+L mediated transport at low homocystine concentrations (Figure 4.5). Thus, the finding of significant arginine uptake with a single transport model best fitting the data, with a rate of uptake and affinity greater than the y^+L alone, was unexpected. We previously noted such residual activity after NEM inhibition (Nel *et al*., 2012) in these cells and it is possible that uptake by y^+ transport may not be completely inhibited at the concentration of NEM used. The sum of residual arginine uptake after inhibition by only leucine or only NEM, appearing to be greater than the total control uptake has been observed in platelets (Leoncini et al., 2003), whereas complete inhibition of lysine uptake was apparent in the presence of NEM plus leucine in erythrocytes (Deves et al. 1993). It is possible, given the complex inhibition of arginine uptake by homocystine, which was not competitive, may affect uptake in a manner similar to other neutral amino acids, such as glutamate, leucine, glutamine, methionine and histidine (Devés and Boyd, 1998), as has been shown for arginine uptake mediated by y^+LAT2/4F2hc transport in *Xenopus laevis* oocytes (Bröer et al. 2000).

The effect of homocystine in the presence of neutral amino acids such as leucine, used to distinguish cationic amino acid transporters (Devés & Boyd; 1998), on arginine uptake was not determined. The approach used in the present study allows the determination of the kinetic constants without using neutral amino acids to distinguish transporters (Nel *et al.*, 2012). Further, the effects of sodium on arginine transport has also been discussed previously (Nel *et al.*, 2012) and were not determined in the present study.

4.3.6 Effect of homocystine on nitric oxide production

Nitric oxide production by HUVEC was greater than that by ECV_{304} cells (Figure 4.6A), and decreased exponentially with increasing exogenous arginine. The rate of NO production by ECV_{304} cells was similar with increasing homocystine concentrations up to 25µM (Figure

4.6B). The rate of NO production was inversely related to the rate of arginine uptake and was similar at homocystine concentrations up to 25µM (Figure 4.6C).

4.4 Discussion

McCully; (1969), after finding evidence of arterial lesions in two children with homocysteinaemia, concluded "....that an elevated concentration of homocysteine, homocystine or a derivative of homocysteine is the common factor leading to arterial damage". Most studies have since shown associations between elevated tHcy and occlusive vascular disease.

The important finding of this *in vitro* study was that arginine uptake by y^+L transport was significantly inhibited by homocystine concentrations of 2.5µM, similar to the concentrations reported in patients with renal disease (Wilcken & Gupta; 1979), in homocystinuric children (Perry *et al.*, 1967), in cystathione-β-synthase deficiency (Hemraj & Griffiths; 1978) and in a single patient with ischaemic heart disease (Wilcken *et al.*, 1983) (see Table 4.1). Furthermore, the inhibition of arginine uptake by y^+L transport could not be reversed even in the presence of high arginine concentrations in either HUVEC or ECV_{304} cells. In contrast, homocystine appeared to increase the rate of arginine uptake by y^+ transport, although with reduced affinity of uptake. Although a previous study has shown that y^+L transport is required for NO production (Arancibia-Garavilla *et al.*, 2003), this *in vitro* study in both HUVEC and ECV_{304} cells suggests that elevated concentrations of homocystine, by inhibiting arginine uptake by y^+L transport, provide a mechanism to explain how increased homocystine may reduce intracellular arginine concentrations. If this were to limit NO production, the study may implicate homocystine as an important risk factor for vascular disease. However, under the particular experimental and assay conditions used in this study, we were unable to demonstrate that homocystine affected the rate of NO production.

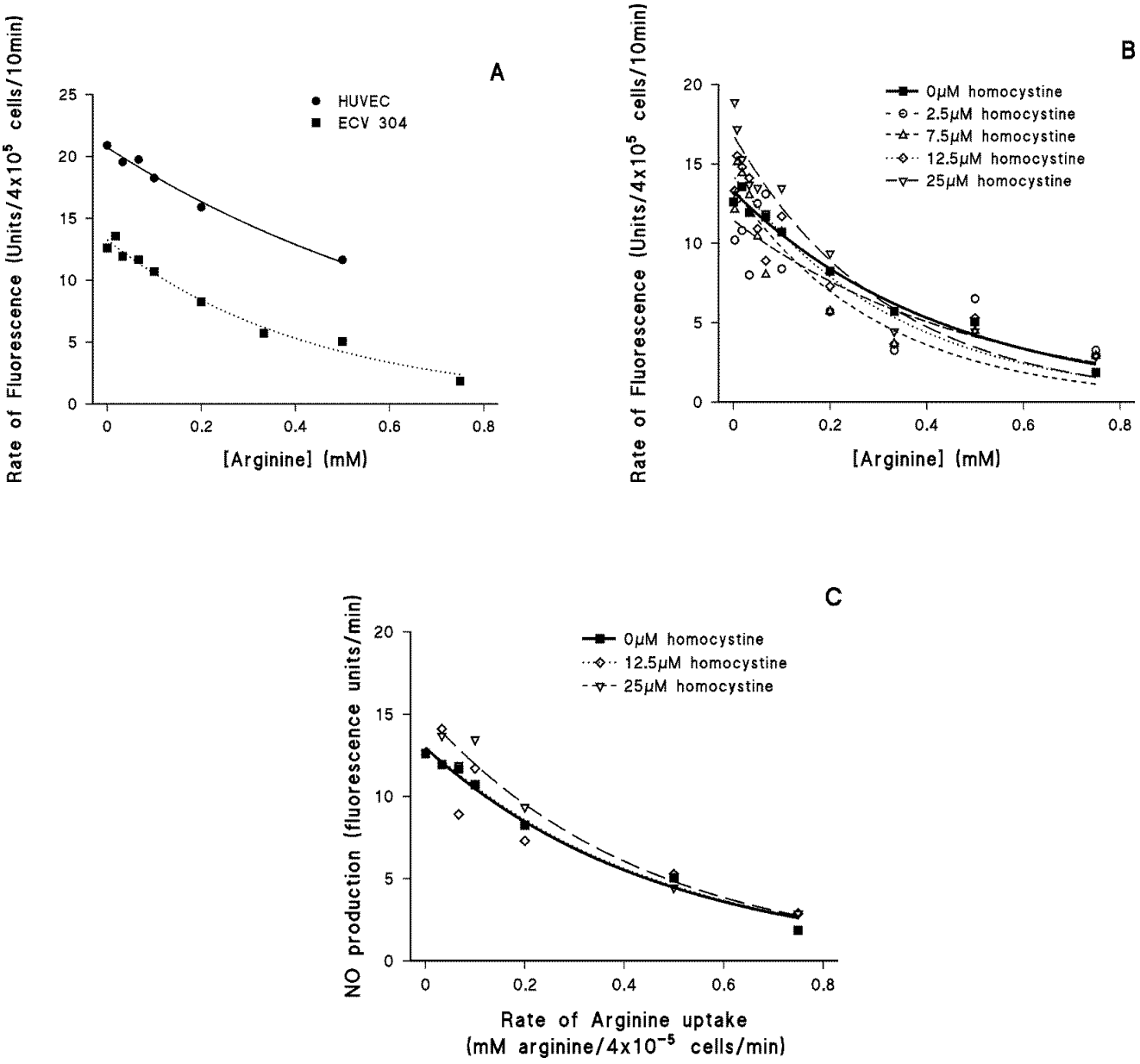

Figure 4.6 Nitric oxide (NO) production by HUVEC and ECV_{304} cells using the fluorescent dye DAF-2, which directly measures NO. **A**. The rate of NO production was greater in HUVEC than ECV_{304} cells and declined with increasing exogenous arginine in ECV_{304} cells; **B**. Homocystine up to 12.5μM had no effect on the rate of NO production. **C.** The rate of NO production declined with increasing rate of arginine uptake (this data was re-worked from Figure 4.1A and Figure 4.6B).

Previous studies have not focused on homocystine, but on the reduced form homocysteine, usually measured as total homocysteine concentration, which was attributed the risk factor for occlusive cardiovascular disease. Homocysteine is taken up by sodium-dependent ASC transport, sharing transport with cysteine, and by L-transport (Ewadh *et al.,* 1990; Jaing *et al.*, 2007) with a K_m*'* between 39μM (Jaing *et al.*, 2007) and 73μM (Ewadh *et al.*, 1990). Few studies have determined the effects of homocysteine on arginine uptake (Jin *et al.*, 2001), while Leoncini *et al.,* (2003) demonstrated that homocysteine concentrations above 25μM significantly inhibit the uptake of arginine non-competitively, into human platelets, in a dose dependent manner. In prolonged experiments (Jaing *et al.*, 2007) determining effects on the uptake of arginine and NO production, rHcy would be converted to other aminothiols, including homocystine, which may impact on NO production (Sengupta *et al.,* 2001).

4.4.1 Homocystine concentrations, transport and effects on nitric oxide production

Homocystine is almost undetectable in plasma of subjects without disease (Perry *et al.*, 1967; Wilcken & Gupta; 1979), whereas in chronic renal failure patients, homocystine concentrations between 1.0±0.7μM (Perry *et al.*, 1967) and 1.7±0.6μM (Wilcken & Gupta; 1979) have been reported. Moreover, homocystine concentrations as high as 7.2±0.2μM have been measured in homocystinuric children. Similarly, in fibroblasts from control subjects, homocystine was undetectable, whereas in fibroblasts from cystathione-β-synthase deficient patients (n=3), homocystine concentrations were 2.4, 7.5±0.2 and 7.9±0.8μM respectively (Hemraj & Griffiths; 1978). Studies since then have only reported total free homocysteine (free reduced + free oxidised homocysteine) which has a concentration of approximately 2μM (Wilcken *et al.*, 1983; Andersson *et al.,* 1993) in control subjects, and may be elevated in patients with stroke (Andersson *et al.*, 1995; Williams *et al.,* 2001; Yang *et al.*, 2004) and end stage renal disease (Wilcken & Gupta; 1979; Sjöberg *et al.,* 2006) (see Table 4.1). However, such total free homocysteine appears to be mostly the cysteine-homocysteine disulphide (Wilcken and Gupta; 1979). Moreover, although chronic renal failure and homocysteinaemic patients are at risk for cardiovascular disease (Wierzbicki; 2007), there appear to be no studies reporting homocystine concentrations in patients with stroke, myocardial infarction and coronary artery disease. Importantly, the present study shows that the elevated concentrations

of 2.5µM homocystine, measured in patient groups at risk of cardiovascular disease as reported in the literature, caused significant inhibition of y^+L transport.

Cusworth and Gattereau (1968), and Foreman *et al.,* (1980) demonstrated that homocystine competes for re-absorption with cationic amino acids in renal cortical tubular uptake via a high affinity system (Foreman *et al.,* 1980). Indirect evidence for such common transport comes from a study in hypercholesterolaemic men supplemented with a high oral dose of 12g/day arginine for 3 weeks, associated with a 10% reduction in plasma tHcy concentration (West *et al.,* 2005), which was inversely proportional to the increase in plasma arginine concentration. In addition, impaired arginine transport has been demonstrated in diseases where tHcy plasma concentrations are often elevated, such as in primary hypertension (Schlaich *et al.,* 2004), heart failure and renal disease (Chin-Dusting *et al.,* 2007). Such impaired transport may account for the endothelial dysfunction observed in these disease states.

Studies with homocystine loading have not been possible because of the low solubility of homocystine (Brenton *et al.,* 1965). However, continuous infusion of homocysteine (0.3g/day/kg body weight) in baboons achieved plasma concentrations of 100-200µM homocystine which induced pre-atherosclerotic or atherosclerotic lesions with collagen fibers, lipid deposition and increased platelet consumption (Harker *et al.,* 1976). Similar findings were observed when hyperhomocysteinaemia was induced by a high methionine diet, which achieved a plasma tHcy concentration of 10.6±2.6µM, without homocystine concentrations being determined in primates (Lentz *et al.,* 1996). Furthermore, the concentrations at which homocystine increased superoxide dependent lipid peroxidation in cultured endothelial cells, were lower than the concentrations of homocysteine which mediated such effects (Heydrick *et al.,* 2004).

The present study demonstrated that homocystine concentrations as low as 2.5µM significantly reduced the theoretical arginine uptake by y^+L transport by approximately 90% in both HUVEC and ECV_{304} cells, without affecting the y^+ tranporter in HUVEC, whilst even increasing y^+ transport in ECV_{304}. As y^+L transport has been demonstrated to play a role in NO synthesis in HUVEC (Arancibia-Garavilla *et al.,* 2003), these data suggest that if y^+L

transport was altered, NO production would be affected. However, although my study provides a mechanism whereby homocystine reduces arginine uptake and may account for some of the detrimental effects attributed to homocysteine, in the cell types and under the experimental conditions used, I was unable to demonstrate impaired NO production. Endothelial cell NO production is determined by the availability of intracellular arginine, determined in part by the recycling of citrulline to arginine (Chin-Dusting *et al.,* 2007). Indeed, endothelial cells are capable of maintaining NO production over a 24 hour starvation period (Mann *et al.,* 2003), and thus our experimental model to determine NO production between 0 – 30 minutes in 5 minute intervals, may not have been appropriate to detect changes in NO production.

4.5 Discussion of study methodology used

We adapted the general non-linear approach of Malo and Bertloot; (1991) to model the effects of homocystine on the kinetics of arginine uptake into HUVEC and ECV_{304} cells by two or more amino acid transporters, with experimental data closely matching the theoretical arginine uptake derived from the calculated constants (Nel *et al.,* 2012). Although the inclusion of a diffusion component and a third transporter (Arancibia-Garavilla *et al.*, 2003) in the model did not improve the fit of the experimental and theoretical data, this does not suggest that these are unimportant, but rather that under the experimental conditions measuring uptake of 10nM L-[^{3}H]arginine, the contribution of diffusion and other transporters was small.

In order to compare the results of the present study to previous studies, I determined the effects of NEM on arginine uptake in the presence of homocystine. The sulphydryl status of membrane proteins plays a key role in facilitating arginine transport, and the present study confirmed that NEM selectively inhibited arginine uptake by y^+ transport (Devés & Boyd; 1998), an energy-independent process (Patel *et al.,* 1996), without affecting arginine uptake by y^+L transport. Although the mechanism is not clear, NEM may directly affect cysteinyl residues or indirectly affect Na^+/K^+-ATPase activity which is required for sodium-dependent transport (Patel *et al.,* 1996). However, membrane potential, which may have influenced arginine uptake at these higher homocystine concentrations was not determined. Furthermore,

when determining arginine uptake following inhibition with NEM, a model of a single transporter best fitted the data, with the rate of uptake and K_m' between those obtained for the y^+L and y^+ transporter. This has been noted previously (Nel et al. 2012) and I hypothesize that NEM may not completely inhibit arginine uptake by y^+ transport.

The present study used DAF-2, previously used to detect extracellular NO (Leikert *et al.*, 2001) production in real time by fluorescence in the supernatant of BAEC (Nakatsubo *et al.*, 1998). When the NO donor NOC-9 was used at 100nM and 200nM, NO was generated while consuming 0.44nM DAF-2, suggesting that the 0.1µM DAF-2 used in the present study to detect NO was not rate limiting (Leikert *et al.*, 2001).

4.6 Study limitations

Homocystine was not measured at concentrations below 2.5µM to determine whether inhibition of y^+L was present within the low concentration ranges reported for normal subjects (Wilcken & Gupta; 1979). The finding that such low concentrations of homocystine nearly abolished y^+L transport was unexpected.

It could be argued that the homocystine used in my arginine uptake assay may have been reduced to homocysteine and thus accounts for the observed effects. However, in the absence of a reducing agent and proteins (Sengupta *et al.*, 2001) on determining arginine uptake over a short 30 second period, this was unlikely. However, in experiments of prolonged incubation of cells in the presence of homocystine, conversion of homocystine to other homocysteine disulphides and protein bound forms (Sengupta *et al.*, 2001) cannot be excluded.

The effect of homocystine in the presence of neutral amino acids such as leucine, previously used to distinguish cationic amino acid transporters (Devés & Boyd; 1998), on arginine uptake or NO production was not determined. The approach used in my study allows the determination of the kinetic constants without using neutral amino acids to distinguish transporters (Nel *et al.*, 2012). The effect of sodium on arginine transport has been discussed

before (Nel *et al.*, 2012), but in the presence of homocystine, the effect of sodium on arginine transport was not determined.

Finally, the study used ECV_{304} cells to model arginine uptake, as it has two arginine transporters (Rotmann *et al.*, 2007) and produces NO, albeit at low levels. As the origin of ECV_{304} is controversial (Brown *et al.*, 2000; Suda *et al.*, 2001), interpretation and extrapolation of the data derived from the ECV_{304} cells to endothelial cells cannot be made and such experiments need to be reproduced in HUVEC.

4.7 Conclusions

Concentrations of homocystine of 2.5µM, similar to those observed in renal failure and homocystinuric patients, nearly abolished arginine uptake by y^+L transport in both HUVEC and ECV_{304} cells. These data provide a mechanism by which homocystine may affect intracellular arginine concentrations especially when extracellular arginine concentrations are low.

CHAPTER 5

The effect of different classes of antihypertensive drugs on arginine uptake, endothelial function and nitric oxide production

5.0 Abstract

Angiotensin enzyme converting inhibitors (ACEI's) have beneficial effects over and above blood pressure lowering effects. In particular, ACEI's appear to enhance NO production which has been suggested to be mediated through reducing oxidative stress. I investigated whether the beneficial effects of ACEI's were mediated, in part, by enhancing the uptake of arginine into cells and thereby increasing NO production. Using the methods described in previous chapters, the initial rate of uptake of L-[^{3}H]arginine was determined in the presence of captopril, enalapril and the diuretic, hydrochlorothiazide (HCTZ). Differences between the ACEI's on uptake of arginine were noted in ECV_{304} cells. At low concentrations, captopril decreased the arginine uptake by y^+L transport, which was not observed with enalapril. At higher concentrations, both ACEI's had bell-shaped dose-response curves. Captopril increased arginine uptake by y^+ transport, whereas enalapril deceased uptake by y^+ transport. The effects of captopril were also noted in HUVEC. Hydrochlorothiazide decreased arginine uptake by y^+L transport, without effects on the y^+ transport. Twenty four hour pre-incubation with the ACEI's prior to determining arginine uptake, also showed differential effects with captopril activating uptake by y^+L transport, and enhanced uptake by y^+ transport. These effects were attenuated with increasing co-incubated arginine concentrations. Enalapril retained the bell-shaped dose-response curve and showed some effect on y^+ transport with increased affinity of uptake (reduced K_m') for both transporters. However, in the model of NO production used, these ACEI's had little effect on NO production and appeared to further reduce the rate of NO production in the presence of increasing arginine. Although the data suggest some enhancement of arginine uptake and transport, the differential effects of ACEI's require further investigation, as do the effects of other classes of antihypertensive drugs.

5.1 Introduction

A number of classes of antihypertensive agents, including beta-blockers; calcium channel blockers; diuretics; ACEI's; angiotensin II receptor blockers; aldosterone receptor antagonists; central acting agents and alpha blockers are currently used to treat hypertension. Although, all of these agents are effective in lowering blood pressure (Chobanian; 2008), they differ in their degree of efficacy (Materson *et al.,* 1993; Ramsay *et al.,* 1999; Chobanian; 2008). Moreover, one particular class of anti-hypertensive agents, namely the ACEI, has been shown to have effects beyond blood pressure lowering (Lonn; 2002).

These blood pressure independent effects, termed pleiotropic effects, are also observed with angiotensin II receptor blockers (Delles *et al.,* 2002). Most other classes of antihypertensive agents have not been shown to have cardiovascular effects which are independent of their blood pressure lowering effects (Schiffrin & Deng; 1995; Delles & Schmieder; 2004). However, the more recently developed third generation $beta_1$ selective blockers (eg. nebivolol) are reported to stimulate NO release (Tzemos *et al.,* 2001), which is thought to be mediated by a reduction in ADMA concentrations in hypertensive patients (Pasini *et al.,* 2008). The pleiotropic effects of the ACEI's and angiotensin II receptor blockers are mainly on the vasculature (Rizzoni *et al.,* 1998; Schiffrin & Deng; 1995). Importantly, ACEI's are responsible for improvements in endothelial function (Buikema *et al.,* 2000) and beneficial in vascular remodelling (Galderisi & De Devitiis; 2008). Moreover, antihypertensive agents which increase the bioavailability of NO may reduce target organ damage in hypertension (Portaluppi *et al.,* 2004).

Having established that ACEI's indeed do have effects beyond blood pressure lowering, a number of investigators explored the potential mechanisms of these effects. In this regard, the beneficial effects of ACEI's on the vasculature have been attributed to the stimulatory effects of ACEI's on eNOS; increased bradykinin and hence NO production; and the ability of ACEI's to scavenge ROS (oxygen free radicals). Indeed, ACEI's have been shown to restore the functioning of eNOS when it has been impaired by increased oxidative stress (Galderisi & De Devitiis; 2008). As a consequence of the stimulatory effects of ACEI's on eNOS, NO

production is enhanced (Balligand *et al.,* 2009). The ability of ACEI's to reduce oxidative stress has been attributed to the sulfhydryl groups, present in some ACEI's, as sulfhydryl groups are known to scavenge superoxide anions (Simic; 1988). Indeed, sulfhydryl containing ACEI's have been shown to produce anti-oxidant effects (Napoli *et al.,* 2004). However, not all ACEI's contain sulfhydryl groups. For example, the ACEI, captopril, contains a sulfhydryl group, but enalapril does not (Jacoby & Rader; 2003; Unger; 2002). Nevertheless, both of these ACEI's have been shown to increase NO production to a similar extent. A study in HUVEC reported that captopril increased NO production by 65% and enalapril by 64% (Desideri *et al.,* 2008). These similar effects of captopril and enalapril on NO production would seem to argue against a major role of the sulfhydryl groups in mediating the beneficial effects of ACEI's on endothelial NO production. Importantly, all types of ACEI's, (ie. sulfhydryl containing or not; lipophilic or not), have been shown to have beneficial effects on endothelial NO production (Desideri *et al.,* 2008; Scribner *et al.,* 2003). The mechanisms of beneficial effects of ACEI's on NO production therefore warrant further investigation.

Bearing in mind that arginine is the substrate for vascular NO production (Kakoki *et al.,* 2004), and hence is an important determinant of NO production especially in the presence of endothelial dysfunction, such as occurs in hypertension (Panza *et al.,* 1995); the activity of arginine transporters may play a role. Indeed, studies have reported decreases in the activity of either y^+ (Yang & Kaye; 2006) or y^+L (Moss *et al.,* 2004) transporters in hypertension. In this regard, ROS have been shown to mediate oxidation of the free sulfhydryl group in the y^+ transporter (Patel *et al.,* 1996), the effects of which would be reduced arginine transport via this transporter. Hence, the anti-oxidant properties of at least the sulfhydryl containing ACEI's may produce beneficial effects on the y^+ transporters. In apparent conflict with these findings, are studies showing that angiotensin II increases the expression and activity of y^+ transporters (Hultström *et al.,* 2009; Low & Grigor; 1995). As ACEI's result in decreased angiotensin II production, it could be argued that non sulfhydryl containing ACEI's (which therefore lack anti-oxidant properties) may reduce arginine transport. Hence, the question remains as to the mechanisms responsible for the increased NO production mediated via ACEI's in the absence of anti-oxidant effects.

Although in endothelial cells, arginine is transported by at least two transporters, y^+ and y^+L (Kakoki *et al.,* 2006, Rottmann), the effects of ACEI on the y^+L (high affinity) transporter have not previously been investigated. In addition, despite reports of decreased uptake of arginine transporters in hypertension (Yang & Kaye; 2006; Moss *et al.,* 2004; Schlaich *et al.,* 2004), the effects of antihypertensive agents on the arginine transporters (y^+ and y^+L) have not previously been investigated. As extracellular arginine concentrations are reported in some studies to be increased in hypertension, due to reduced transport (Moss *et al.,* 2004; Perticone *et al.,* 2005); and/or amino acids such as ADMA (Perticone *et al.,* 2005) which compete for the same transporters, the potential effects of ACEI's and other antihypertensive agents on arginine transporters warrant investigation. Hence, the aim of this part of my thesis was to investigate the effects of a sulfhydryl versus a non-sulfhydryl containing ACEI, as well as the effects of another class of antihypertensive agent (without pleiotropic effects), the diuretic, HCTZ, on arginine transport by the y^+ and y^+L transporters.

5.2 Materials and methods

For this part of the study, the primary HUVEC were used in addition to the immortalized ECV_{304} cells. The HUVEC and their specialized culture medium, EBM-2®-2 (Clonetics®, Lanzor) was obtained from Whitehead Scientific, Brackenfell, SA. The captopril, enalapril, and HCTZ were obtained from Sigma-Aldrich, Chemie, GmbH.

The cell culture methods for ECV_{304} and HUVEC were as described in sections 2.2.1 and 2.2.3 respectively, and illustrated in figure 2.2 of chapter 2. The kinetics of uptake of arginine was determined by measuring the initial rate of uptake of 10nM L-[^{3}H]arginine over 30 seconds in the presence of the drug with unlabelled arginine in PBS as described in sections 2.3; 2.3.1 and figure 2.2 of chapter 2. The concentrations of the antihypertensive drugs were used at clinical dosages of 12.5µM - 25µM with data obtained between 0µM - 250µM.

After ECV_{304} cells were incubated for 30seconds with 0-100µM arginine only, 2.5-100µM specific drug only or a combination of arginine in the concentration range of 0-100µM and one of the antihypertensive/cardiovascular drugs at a concentration range of 2.5-100µM, data were

obtained from radioactive arginine (L-[^{3}H]arginine) uptake counts using a scintillation counter. The arginine uptake rate constants were calculated as described in detail in Chapter 3.

5.3 Results

5.3.1 Effects of antihypertensive drugs on initial rates of uptake of arginine

For uptake of L-[^{3}H]arginine by ECV_{304} cells, low concentrations of captopril decreased both V_{maxa} and K_{ma} of the y^+L transporter at 2.5μM ($p<0.05$), with a bell shaped dose-response curve above these concentrations, reaching a maximum at 33μM captopril. Captopril increased both V_{maxb} and K_{mb} of the low affinity/high rate transporter (y^+ transporter), with lower activity at 25μM captopril (Figure 5.1). Similarly, captopril decreased arginine uptake by y^+L transport (V_{maxa}) in HUVEC (captopril 12.5μM: $p=0.0024$; 25μM: $p=0.0095$) whereas y^+ transport (V_{maxb}) was unchanged when the concentration of captopril was 12.5μM and was increased when captopril was 25μM ($p=0.0019$; Table 5.1). Captopril did not affect the K_{ma} or K_{mb} for y^+L or y^+ transport respectively in HUVEC (Table 5.1).

Enalapril also demonstrated bell shaped rate of uptake for the y^+L-transporter, whereas y^+ transport was decreased up to 100μM enalapril, but increased above this concentration (Figure 5.2). Increasing concentrations of HCTZ decreased the rate and affinity of uptake by y^+L transport, whereas y^+ transport was unaffected (Figure 5.3).

5.3.2 Pre-incubation of ECV_{304} cells with antihypertensive drugs

Twenty four hour pre-incubation of 12.5μM captopril showed activation of the y^+L transporter in ECV_{304} cells as noted in the Michaelis-Menten rate plot (Figure 5.4), for total arginine uptake calculated after correcting for dilution. Increasing concentrations of co-incubated arginine attenuated the activation of total uptake from arginine concentrations between 67μM to 300μM arginine. The inverse of this total uptake plotted as a Lineweaver - Burke reciprocal

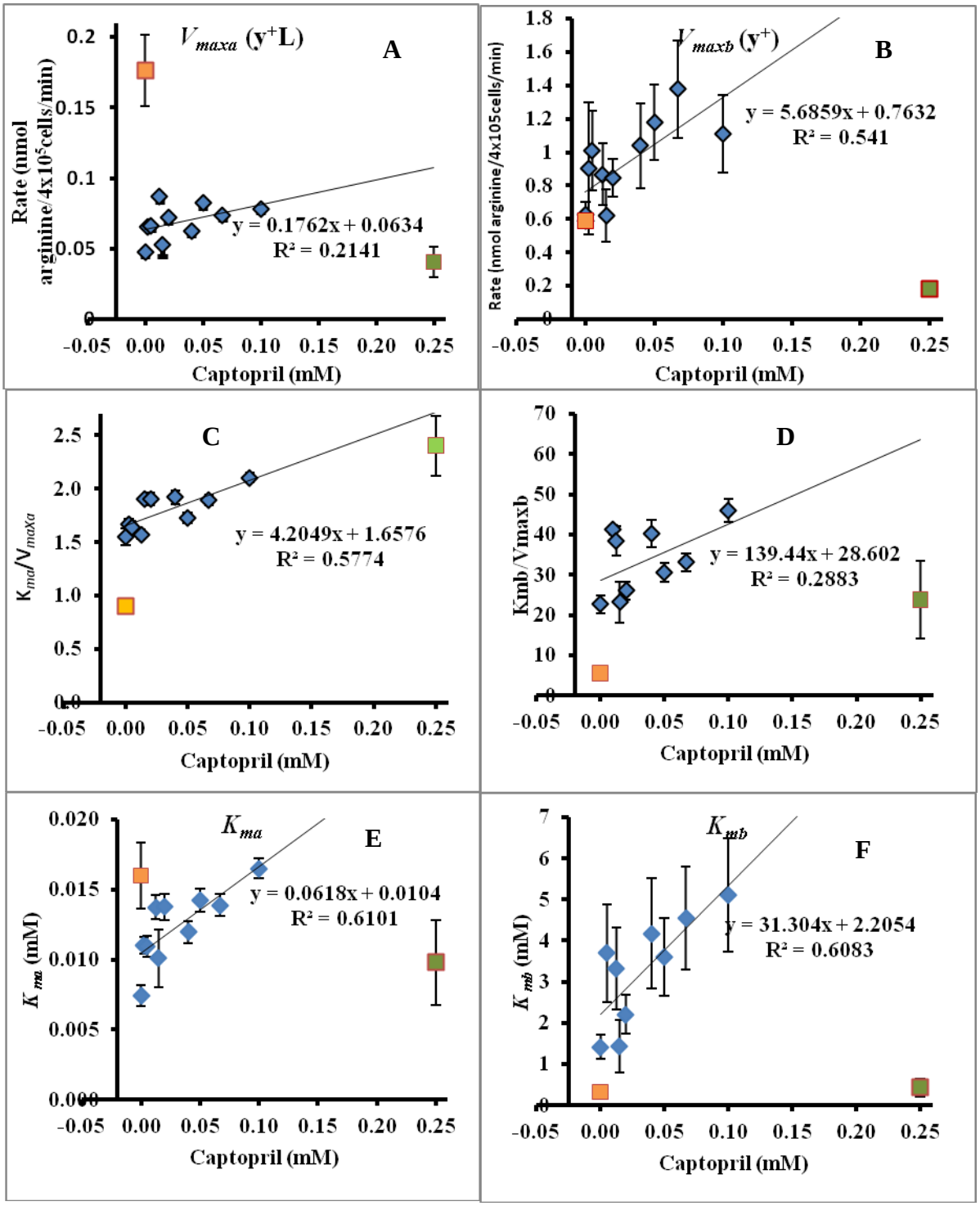

Figure 5.1 Effect of the angiotensin-converting enzyme inhibitor, captopril, on the initial rate kinetic parameters, A) V_{maxa}, C) K_{ma}, and B) V_{maxb}, D) K_{mb} and E) and F) the ratio of K_m/V_{max} on arginine uptake by y^+L ('a') and y^+ ('b') transporter respectively. ■ Show data at baseline without captopril and ■ show data at baseline with 0.25mM captopril.

Table 5.1 Effects of captopril on the kinetic constants of L-[^{3}H]arginine uptake by HUVEC. *p<0.05/** p<0.005 *vs* captopril=0mM. Differences between uptake in the presence of 0.0125mM and 0.025mM captopril were not significant. The high SEM for V_{maxa} was due to the experiments having data points with low arginine concentrations.

	Captopril 0mM	Captopril 0.0125mM	Captopril 0.025mM
V_{maxa} (nM/4x10^5 cells/min)	0.106±0.025	0.012±0.016*	0.017±0.013 (p=0.059)
K_{ma} (mM)	0.018±0.004	0.014±0.017	0.024±0.014
V_{maxb}(nM/4x10^5 cells/min)	0.933±0.077	1.143±0.083	1.258±0.037**
K_{mb} (mM)	0.647±0.170	0.516±0.093	0.545±0.047

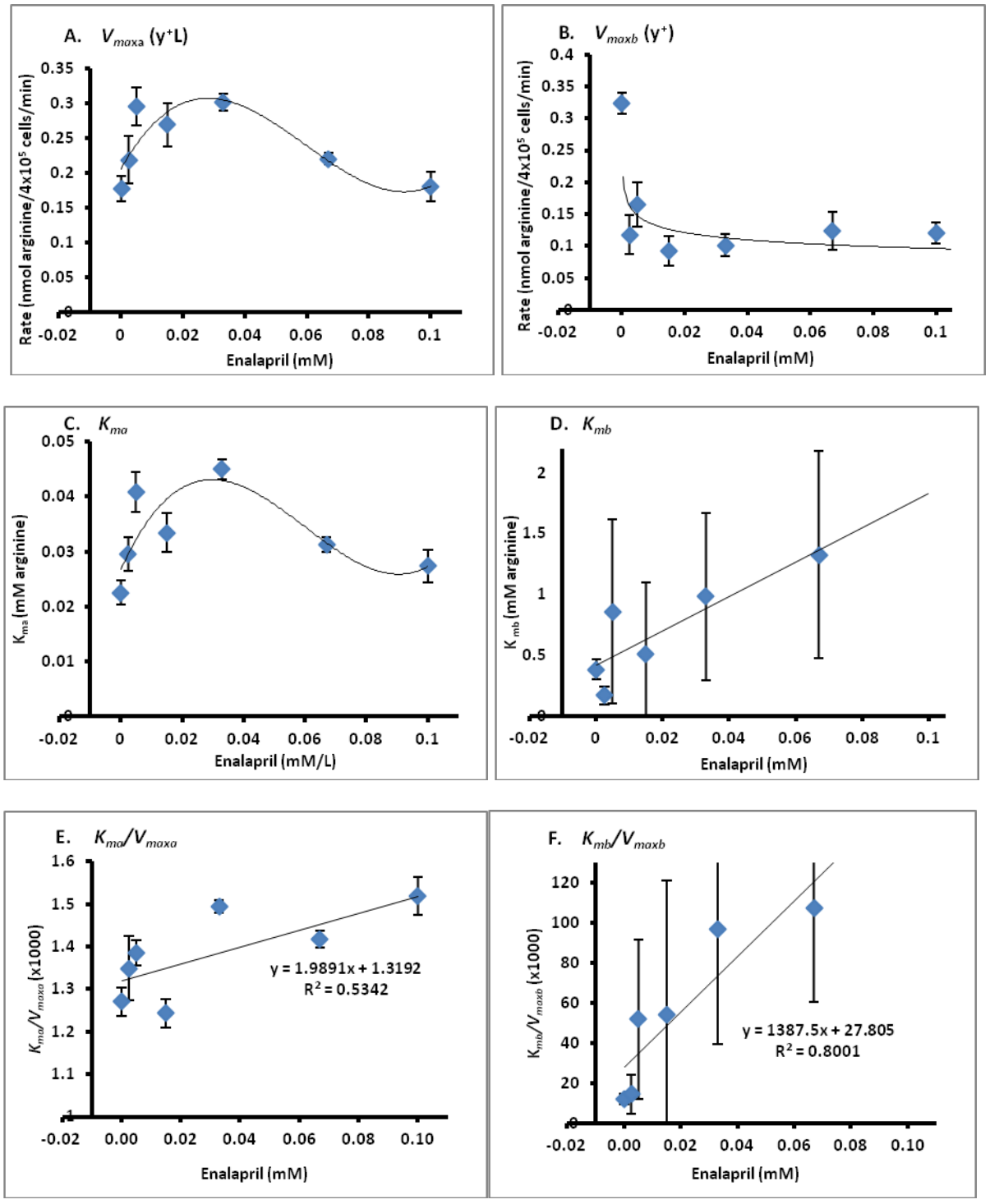

Figure 5.2 Effect of the angiotensin-converting enzyme inhibitor, enalapril, on the kinetic parameters, A) V_{maxa}, B) V_{maxb}, C) K_{ma}, D) K_{mb} and the ratio E) and F) K_m/V_{max} on uptake by y^+L and y^+ transporter respectively.

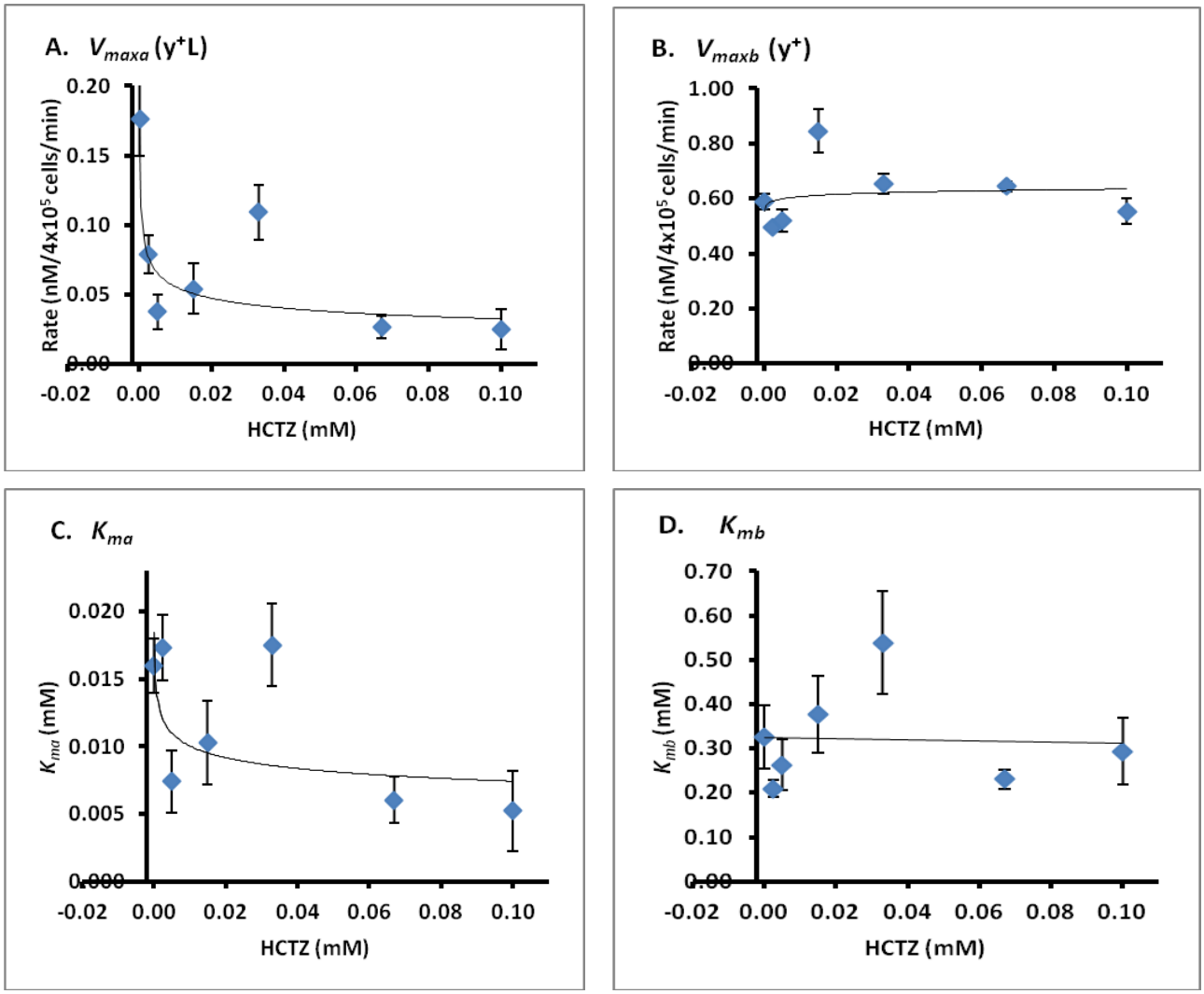

Figure 5.3 Effect of HCTZ on the kinetic constants of L-[^{3}H]arginine uptake into ECV_{304} cells. Uptake was measured in the presence of HCTZ and unlabelled arginine as described in the text. A) V_{maxa} decreased with increasing concentration of HCTZ, whereas B) HCTZ had no effect on V_{maxb} C) K_{ma} and D) K_{mb} increased up to 0.033mM HCTZ and were decreased above these concentrations.

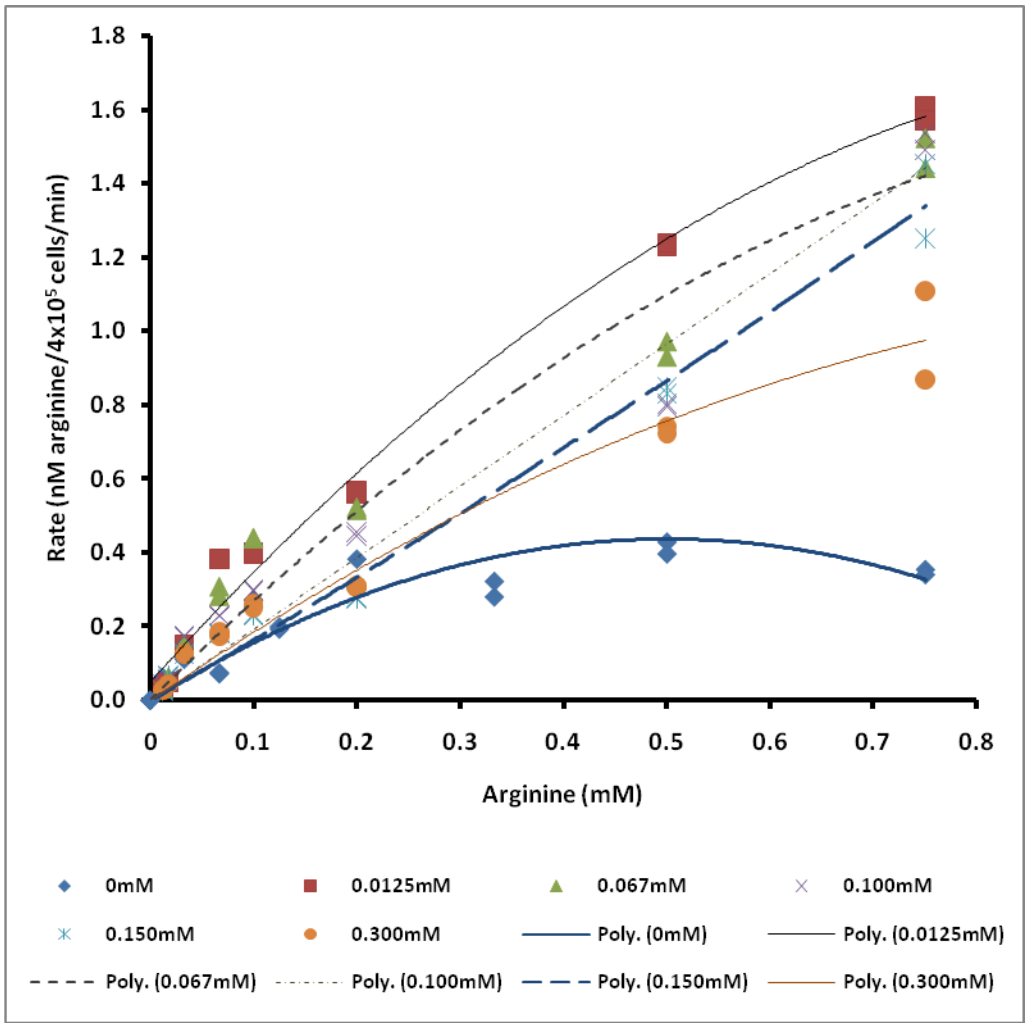

Figure 5.4 Dilution corrected uptake of L-[^{3}H]arginine by ECV_{304} cells showing increased arginine uptake by the cells following 24hour pre-incubation with the ACEI, captopril and attenuation of this effect when the cells were co-incubated with unlabelled arginine.

graph, revealed activation of a high affinity component of uptake (Figure 5.5A) without apparent differences between the concentrations of co-incubated arginine. Although the low-affinity/high rate transport component showed increased uptake, the activation of uptake was not apparent, i.e. no upward curvature of the plot (Figure 5.5B). The kinetic constants were not calculated as the mode of activation (not linear) needs to be determined to modify the model of Malo and Berteloot (1991).

Activation of uptake was not noted when ECV_{304} cells were pre-incubated with 25μM enalapril. The V_{maxa} increased with a bell-shaped dose-response curve and a significantly decreased K_{ma}. The V_{maxb} was decreased in the presence of 67μM and 100μM co-incubated arginine, with K_{mb} significantly decreased (Figure 5.6).

5.3.3 Effect of captopril and enalapril on nitric oxide production by ECV_{304} cells

Both captopril and enalapril further attenuated the decrease by added unlabelled arginine on the rate of NO production by ECV_{304} cells (Figure 5.7).

5.4 Discussion

Of the many drugs used to treat hypertension, the ACEI have been shown to have effects beyond blood pressure lowering (Lonn; 2002), improving endothelial function (Buikema *et al.*, 2000), by beneficially remodelling of the vasculature (Galderisi and De Devitiis; 2008), lowering the production rate of superoxide anions, even when angiotensin II is not elevated (Rajagopalan *et al.*, 1996), and importantly by improving endothelial NO production (Desideri *et al.*, 2008; Scribner *et al.*, 2003). Indeed, antihypertensive agents which increase the bioavailability of NO may reduce target organ damage in hypertension (Portaluppi *et al.*, 2004) and as such, ACEI's may be able to reverse endothelial dysfunction.

The sulfhydryl group of ACEI's, such as captopril, may confer additional beneficial anti-oxidant properties (Simic; 1988). Indeed, the improvement of the NO pathway by the sulfhydryl containing ACEI, zofenopril, has been shown to be due to a reduction in oxidative

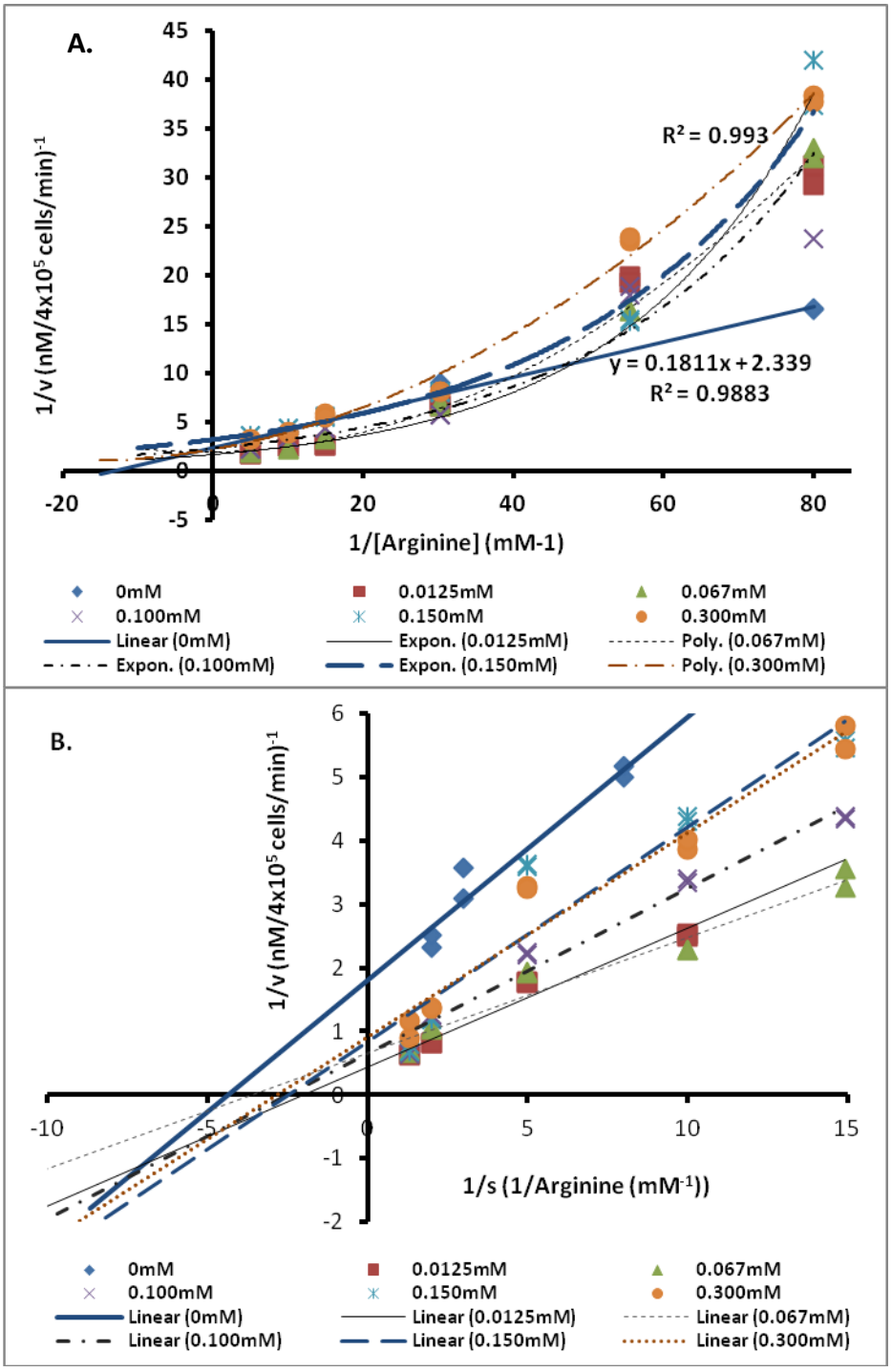

Figure 5.5 ECV_{304} cells were pre-incubated with captopril (0.0125mM) with various concentrations of unlabelled arginine for 24 hours prior to determining 10nM L-[^{3}H]arginine uptake as described in the text. The Lineweaver-Burk plot of A) the high affinity/low uptake rate component (y^+L) of arginine uptake shows activation of uptake in the presence of captopril, whereas this was not apparent for B) the low affinity/high uptake rate component (y^+).

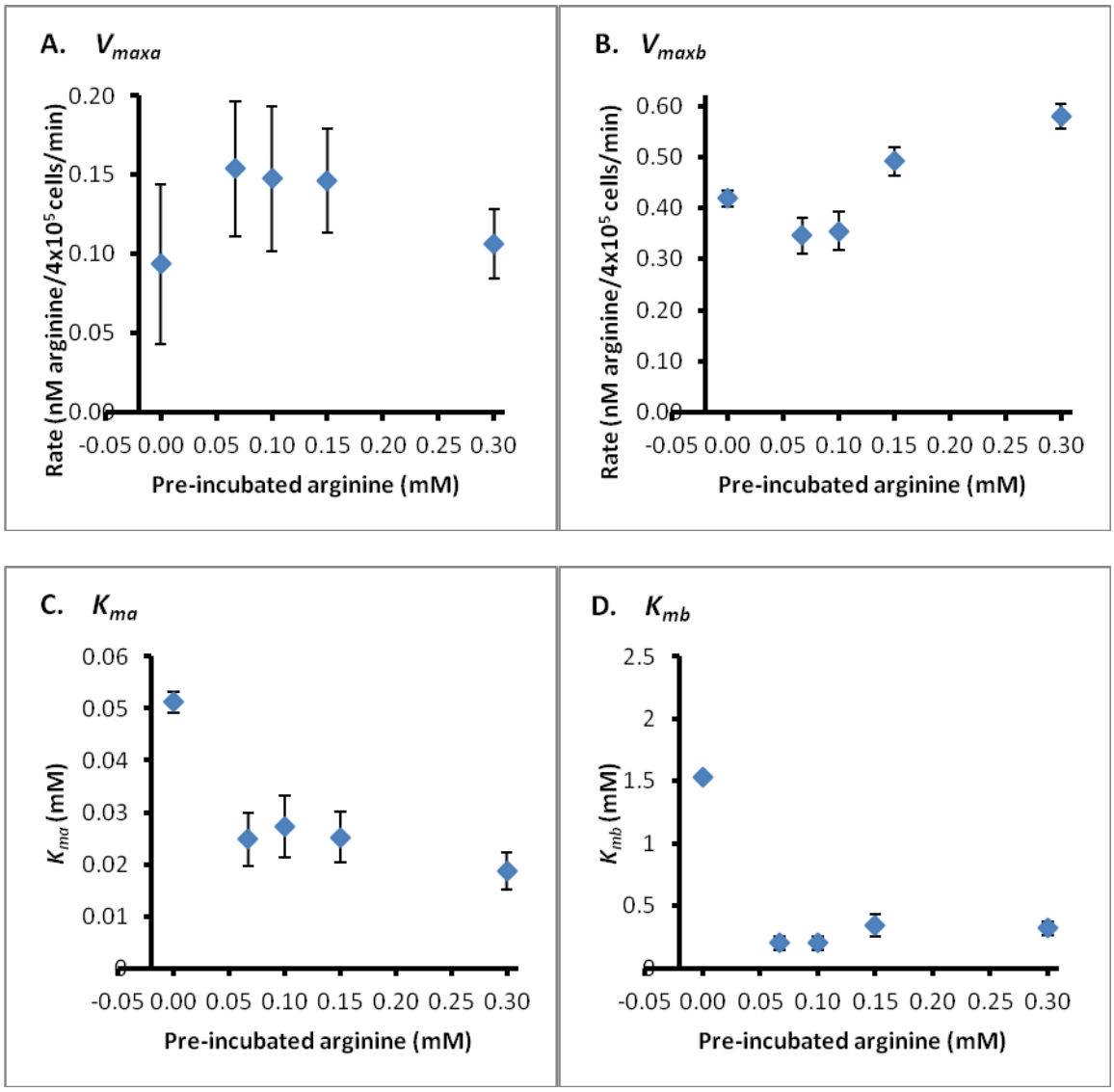

Figure 5.6 Effect of 24-hour pre-incubation with 0.025mM enalapril, on L-[^{3}H]arginine uptake of ECV_{304} cells A) V_{maxa} showed a bell shape response whereas B) V_{maxb} changed with increasing concentrations of co-incubated unlabelled arginine. Both C) K_{ma} and D) K_{mb} were significantly decreased with co-incubated arginine.

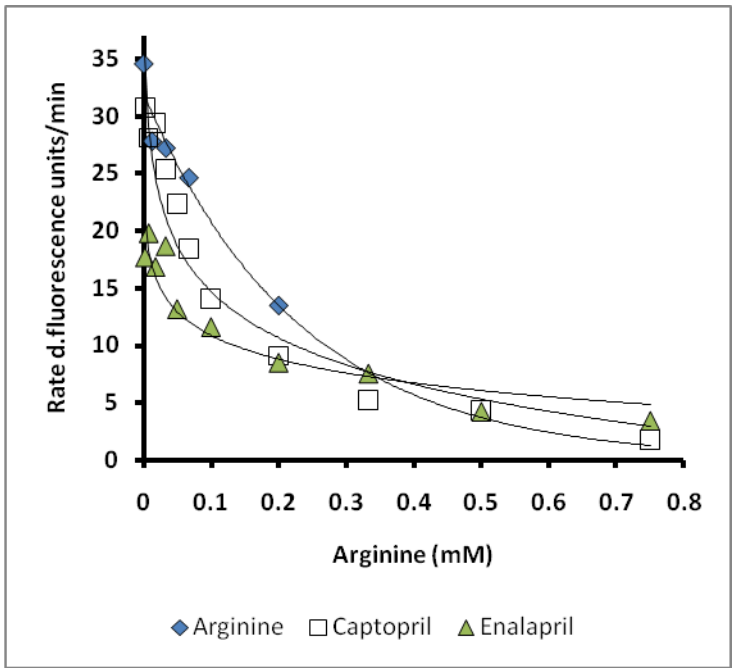

Figure 5.7 Effect of captopril and enalapril on the rate of nitric oxide production by ECV_{304} cells in the presence of exogenously added arginine. Nitric oxide production was expressed as the rate of DAF-2 fluorescence per minute. Measurements were done in duplicate.

stress in primary hypertensive patients (Napoli *et al.*, 2004). However, zofenopril, but not captopril or enalapril, improved NO production in BAEC (Scribner *et al.*, 2003), suggesting that even different sulfhydryl containing ACEI's may mediated their effects by different mechanisms.

As far as I can determine, the data reported in this chapter are the first to determine whether ACEI's function directly affect arginine uptake by cationic transport. The data showed an initial decrease in the rate of L-[^{3}H]arginine uptake for captopril, but not for enalapril, followed by bell-shaped dose-response curves for the high affinity/low activity (y^+L) transporter for both ACEI's. However, differences were noted between the effects these ACEI's had on the kinetic constants of uptake by the low affinity/high activity (y^+) transporter with captopril increasing activity, but enalapril having little effect, indicating the possible importance of the sulphydryl group. In contrast to the data for the ACEI's, HCTZ appeared to decrease arginine uptake by y^+L transport, without affecting the y^+ transport.

Further differences between captopril and enalapril were apparent when ECV_{304} cells were incubated with these ACEI's in the presence of arginine for extended periods. Captopril activated arginine uptake by y^+L transport and added arginine attenuated the enhanced uptake by y^+ transport. In contrast such activation was not apparent with enalapril but added arginine increased the affinity of both the y^+L and y^+ transport. The differences between the initial rate of uptake and the long term pre-incubation studies suggest that captopril exerts additional effects on the cells, independent of the direct effects on the intial rates of uptake. This may be through increasing activity or expression of the transporters. Indeed, there have been reports of angiotensin II increasing the expression and activity of y^+ transporters (Low and Grigor; 1995, Hultström *et al.*, 2009), which would be in apparent conflict with the present data showing increased arginine transport by captopril and enalapril.

A limitation of the present study is that the model of Malo and Berteloot (1991) does not readily accommodate activation of uptake, for example by using an allosteric sigmoid fit to the y^+L transporter data. However, activation of uptake was readily apparent from the Lineweaver-Burk plots. Furthermore, as discussed previously in Chapter 4, despite enhanced

uptake of L-[^{3}H]arginine, the anticipated increase in NO production could not be demonstrated in the experimental setup used. Finally, the data has been limited to two ACEI's and HCTZ, but effects of other classes of antihypertensive drugs on arginine uptake need to be determined.

5.5 Conclusions

The observed transporter activation effect by captopril and to a lesser extent by enalapril, may suggest a drug class effect as such effects were not observed with HCTZ. The increased arginine uptake and activation of arginine membrane transporters in the presence of ACEI's, could partly explain the beneficial protective effects of the ACEI's, which may be mediated either by increasing arginine uptake when the arginine concentrations are low or perhaps by increasing the activity and/or expression of arginine transporters. The differential effects of the ACEI's on the transporters require further elucidation, and the effects of other anti-hypertensive drug classes need to be determined.

CHAPTER 6

6.1 Summary and conclusions

Cardiovascular diseases are one of the leading causes of death worldwide, with the treatment of such diseases costing billions of dollars. As hypertension remains one of the major risk factors for cardiovascular disease, a better understanding of the mechanisms responsible for hypertension are required. One of the potential causes of hypertension is endothelial dysfunction associated with a decrease in NO production.

However, concentrations of the NO precursor, arginine, are elevated in hypertension, yet, arginine supplementation has been shown to lower blood pressure. A possible explanation for this paradox is that membrane transport of arginine may be implicated in hypertension, as limited uptake of arginine into endothelial cells may decrease NO production. In order to address these issues, endothelial cell transport of arginine needs to be characterised, and substances which may inhibit or enhance arginine uptake by these transporters need to be determined.

To address these questions, I set up the assay to measure L-[^{3}H]arginine uptake using ECV_{304} cells, which grow readily and express the two transporters facilitating arginine uptake into cells. The theoretical curves of uptake were derived from the kinetic constants determined from dilution corrected data, linearized either as Lineweaver-Burke or Eadie-Hofstee plots. The data from either plot yielded the same value for each constant. However, it was readily apparent that the theoretical plot significantly overestimated the experimental data. Few studies in the literature used only the labelled amino acid to determine constants and such an approach was expensive and would produce significant radioactive waste. The difficulty is that at least two transporters mediate uptake into cells and thus the components of the linearized plots represent the sum of such uptake, not that of the contributions of the individual transporters. Despite this serious error in earlier studies, the yielded constants provided initial estimates for subsequent non-linear modelling and the Lineweaver-Burke plots provided some indication of what type of inhibition might be anticipated.

Furthermore, the methods used by other researchers frequently examined particular concentrations of substrates and substances which modify cationic amino acid uptake such as leucine. Sodium and leucine have been used extensively to distinguish between the y^+ and the y^+L transporters. However, as far as I could determine, the mathematical models have only determined competitive effects of leucine on lysine or arginine uptake by cells. The equations for non-competitive or uncompetitive inhibition have not been utilized to determine if such models improved the fit of the experimental data. Thus alternative methods were needed to calculate the constants.

The raw data yielded by the uptake curve is the uptake of only the trace L-[^{3}H]arginine. Unlabelled arginine competitively inhibits uptake, rapidly decreasing uptake in a non-linear manner, dependent on the concentrations of both the labelled and unlabelled amino acid. Therefore, the concentrations of both need to be included in calculations to determine the kinetics of uptake. The method of Malo and Berteloot (1991) provided such a model describing uptake of glucose by more than one transporter. The rapid kinetics of glucose uptake required the Malo and Berteloot group of scientists to develop a filtration device to stop uptake. Fortunately, the uptake of amino acids appears to be an order of magnitude slower than that of glucose and such a device did not appear to be necessary for the present studies. It should be noted that Graphpad Prism was particularly useful in providing the statistical data as well as in comparing values of constants. The program requires relatively stable data in order to provide means with small SEM's.

The kinetic constants derived from the equation agreed closely with the experimental data and allowed uptake by the individual transporters to modelled. In the absence of selective inhibitors for either transporter, such a method may be useful for elucidating the role of the individual transporters in arginine uptake. Although initially the equations for competitive, as well as non- and un-competitive inhibition were included and tested, the method allows for simply observing changes in the V_{max}', K_m' as well as the ratio K_m'/V_{max}' to determine the model of inhibition. The K_i' could then be determined for the relevant transporter. The literature does have several methods for determining K_i' when the intercepts do not lie on the axes. The method and nomenclature used by Krupyanko; (2007) may not be universally

accepted, but provide useful descriptions and equations for observed changes in V_{max}' and K_m'. Using such approaches, I was able to show that leucine may not affect arginine transport competitively.

However, because of the very different approach used, whether the individual transport is consistent to that ascribed to y^+L and y^+ transport, remains to be demonstrated. Furthermore, it must be noted that although K_m values are relatively stable, V_{max} values are sensitive to experimental variation. Although this method may provide useful data, other methods based on membrane transport principles may yield additional useful data. Indeed, although the method allowed for inhibition to be modelled, modelling of the activation of transport requires further investigation.

Having developed the methodology above, the next step was to determine which factors may affect arginine transport. Total homocysteine concentrations are a well described risk factor for occlusive vascular disease and vitamin supplementation studies have resulted in significantly reduced tHcy concentrations. However, large supplementation trials in patients with pre-existing cardiovascular disease were unable to demonstrate reductions in subsequent cardiovascular events. In the absence of a clear mechanism(s) as to the action of reduced homocysteine on endothelial function, it has been suggested that homocysteine is a marker and not a causal risk factor of cardiovascular disease. Indeed, the early studies used, and measured, the disulphide form of homocysteine, homocystine. Moreover, it was indeed homocystine, which was shown to reduce cationic amino acid re-uptake in the proximal tubules and cause the significant atherosclerotic plaques in chronically homocystine infused baboons.

Homocystine is undetectable in healthy individuals, with concentrations of <10µM homocystine measured in patients at risk for occlusive vascular disease, including renal dialysis patients and homocystinuric children. My study demonstrated that homocystine nearly abolished L-[^{3}H]arginine uptake into both ECV_{304} cells and HUVEC by y^+L transport at concentrations of 2.5µM, which although low, are within the high range for patients at risk for occlusive disease. My study provides *in vitro* data which would support studies to measure

homocystine concentrations in patients with cardiovascular disease and to correlate these with the severity of the disease, providing a possible mechanism as to how homocystine may reduce arginine uptake into endothelial cells. However, it still needs to be demonstrated, in an appropriate model, that homocystine reduces NO production by these cells.

Having observed the effect of sulphydryl groups such as in NEM and the reports of homocysteine on arginine uptake, I considered whether the sulphydryl containing ACEI, captopril, or indeed other ACEI's or other cardiovascular drugs affected arginine transport. Differences between the captopril and enalapril in the rates of uptake and affinity of arginine uptake for the two transporters were noted in ECV_{304} cells. The effects of captopril were also demonstrated in HUVEC. The enhancements of uptake observed with the ACEI's were not apparent for HCTZ where arginine uptake by y^+L transport was inhibited without effects on y^+ transport. A twenty four hour pre-incubation with the ACEI's prior to determining uptake also showed differential effects with captopril activating uptake by y^+L transport and enhanced uptake by y^+ transport. These effects were attenuated with increasing co-incubated arginine concentrations. Enalapril retained the bell-shaped dose-response curve and showed some effect on y^+ transport with increased affinity of uptake (reduced K_m') for both transporters. However, in the model of NO production used, these ACEI's had little effect on NO production and appeared to further reduce the rate of NO production in the presence of increasing arginine. Although the data suggest some enhancement of uptake of arginine transport, the differential effects of ACEI's require further investigation as do the effects of other classes of cardiovascular drugs.

In summary, the thesis comprises a body of work where 1) a model of uptake for two or more transporters was adapted to model arginine uptake into both ECV_{304} cells and HUVEC, 2) using the developed model, homocystine was shown to nearly abolish arginine uptake by y^+L transport and 3) the ACEI's, enhanced arginine transport.

6.2 Recommendations and future research

Supplementation with arginine in hypertensive patients is not recommended because of the known adverse effects that supraphysiological arginine concentrations have on the kidneys. Importantly, since arginine concentrations are often normal or even elevated in hypertensive patients, indicating another cause for the hypertension such as inefficiency of an arginine membrane transporter, increasing arginine concentrations by supplementation would not decrease the patient's blood pressure. In this regard, since homocystine inhibits the uptake of arginine by the endothelial cell membrane transporters, homocystine would decrease the uptake of the NO pre-cursor, arginine, resulting in futher dysfunction of the vascular endothelium and thus increase the blood pressure even further. For these reasons, both arginine and/or homocysteine would be contra-indicated in hypertension.

Future hypertension treatment research should focus on improving the functionality and effectiveness of the transport of arginine into vascular endothelial cells. This could possibly be achieved by the development of new anti-hypertensive drugs having novel mechanisms of action on the transporters of arginine.

APPENDICES

APPENDIX A

STANDARD KREBS-HENSELEIT BUFFER SOLUTION (Na^+ -Krebs) (Arancibia-Garavilla *et al*., 2003) with a total Na^+ concentration of 157mM as used in arginine uptake experiments as described in Chapter 3.

NaCl	131mM
KCl	5.6mM
$NaHCO_3$	25mM
NaH_2PO_4	1mM
HEPES	20mM
$CaCl_2$	2.5mM
$MgCl_2$	1mM
pH	7.4 at 37ºC

MODIFIED KREBS-HENSELEIT BUFFER SOLUTION (Arancibia-Garavilla *et al*., 2003) with choline chloride replacing NaCl to a total Na^+ concentration of 26mM to test the Na^+ dependence of arginine uptake as described in Chapter 3.

Choline Chloride	131mM
KCl	5.6mM
$NaHCO_3$	25mM
NaH_2PO_4	1mM
HEPES	20mM
$CaCl_2$	2.5mM
$MgCl_2$	1mM
pH	7.4 at 37ºC

MODIFIED KREBS-HENSELEIT BUFFER SOLUTION (Arancibia-Garavilla *et al*., 2003) with choline chloride replacing NaCl and $KHCO_3$ and KH_2PO_4 replacing $NaHCO_3$ and NaH_2PO_4 respectively, in order to have a Na^+ free buffer to test the Na^+ dependence of arginine uptake as described in Chapter 3.

Choline Chloride	131mM
KCl	5.6mM
$KHCO_3$	25mM
KH_2PO_4	1mM
HEPES	20mM
$CaCl_2$	2.5mM
$MgCl_2$	1mM
pH	7.4 at 37ºC

MODIFIED KREBS-HENSELEIT BUFFER SOLUTION (Arancibia-Garavilla *et al*., 2003) with an increased Ca^{2+} concentration to obtain optimal results for the fluorescent DAF-2 NO sensitive dye, as recommended by the suppliers (Sigma-Aldrich, Chemie, GmbH) in a personal e-mail communication, as used in the DAF-2 NO detection method described and used in Chapters 2, 4 and 5.

KCl	6mM
Na_2HPO_4	1mM
NaH_2PO_4	0.2mM
$MgSO_4$	1.4mM
NaCl	128mM
HEPES	10mM
Glucose	5.5mM
Pyruvic acid	2mM
$CaCl_2$	1mM
pH	7.4 at 37°C.

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HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
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CLEARANCE CERTIFICATE	**M120154**
PROJECT	Determination of Cellular L-arginine Transport (Previously M030935)
INVESTIGATORS	Dr Marietha Nel.
DEPARTMENT	Pharmacy & Pharmacology
DATE CONSIDERED	Ad hoc
DECISION OF THE COMMITTEE*	Renewal Approved

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07/02/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

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