Diminished L-arginine bioavailability in hypertension

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ABSTRACT

L-Arginine is the precursor of NO (nitric oxide), a key endogenous mediator involved in endothelium-dependent vascular relaxation and platelet function. Although the concentration of intracellular L-arginine is well above the $K_m$ for NO synthesis, in many cells and pathological conditions the transport of L-arginine is essential for NO production (L-arginine paradox). The present study was designed to investigate the modulation of L-arginine/NO pathway in systemic arterial hypertension. Transport of L-arginine into RBCs (red blood cells) and platelets, NOS (NO synthase) activity and amino acid profiles in plasma were analysed in hypertensive patients and in an animal model of hypertension. Influx of L-arginine into RBCs was mediated by the cationic amino acid transport systems $y^+$ and $y^+$L, whereas, in platelets, influx was mediated only via system $y^+$L. Chromatographic analyses revealed higher plasma levels of L-arginine in hypertensive patients (175 ± 19 µmol/l) compared with control subjects (137 ± 8 µmol/l). L-Arginine transport via system $y^+$L, but not $y^+$, was significantly reduced in RBCs from hypertensive patients (60 ± 17 µmol·l$^{-1}$·cells$^{-1}$·h$^{-1}$; n = 16) compared with controls (90 ± 17 µmol·l$^{-1}$·cells$^{-1}$·h$^{-1}$; n = 18). In human platelets, the $V_{max}$ for L-arginine transport via system $y^+$L was 86 ± 17 pmol·10$^9$ cells$^{-1}$·min$^{-1}$ in controls compared with 36 ± 9 pmol·10$^9$ cells$^{-1}$·min$^{-1}$ in hypertensive patients (n = 10; P < 0.05). Basal NOS activity was decreased in platelets from hypertensive patients (0.12 ± 0.02 pmol/10$^8$ cells; n = 8) compared with controls (0.22 ± 0.01 pmol/10$^8$ cells; n = 8; P < 0.05). Studies with spontaneously hypertensive rats demonstrated that transport of L-arginine via system $y^+$L was also inhibited in RBCs. Our findings provide the first evidence that hypertension is associated with an inhibition of L-arginine transport via system $y^+$L in both humans and animals, with reduced availability of L-arginine limiting NO synthesis in blood cells.

INTRODUCTION

NO (nitric oxide) is a gaseous free radical formed from the cationic amino acid L-arginine by a family of NOSs (NO synthases), leading to the generation of NO and L-citrulline [1,2]. NO plays an important role in all stages of platelet activation by inhibiting adhesion, secretion and aggregation and by stimulating disaggregation of

Key words: arginine transport, citrulline, hypertension, platelet, red blood cell, system $y^+$L.

Abbreviations: ACE, angiotensin-converting enzyme; NEM, N-ethylmaleimide; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; iNOS, inducible NOS; RBC, red blood cell; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto.

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Platelets express both iNOS (inducible NOS) and eNOS (endothelial NOS) isoforms of NOS, and platelet NOS is activated during platelet adhesion stimulated by collagen and aggregation induced by ADP [3,4]. Although the synthesis of NO by RBCs (red blood cells) remains controversial, several studies suggest that RBCs possess both iNOS and eNOS, and that NO modulates RBC membrane fluidity and aggregability [5–7]. RBCs have also been reported to release NO bound to haemoglobin into the microcirculation under low oxygen tension [8].

Hypertension is characterized by impaired endothelium-dependent vasodilatation, which correlates with an elevation in systemic blood pressure [9–13]. Diminished bioavailability of NO has been implicated as a possible mechanism for endothelial dysfunction in both animal models of hypertension and essential hypertensive patients [9–17]. In contrast, some studies in animal models suggest that NO synthesis is increased, rather than decreased, in hypertension [14].

Platelets from hypertensive patients show increased activation associated with a disturbance of the L-arginine/NO pathway [18–20]. RBCs from hypertensive patients exhibit reduced membrane fluidity, leading to increased blood viscosity, platelet aggregation and peripheral vascular resistance [21].

In hypercholesterolaemia and atherosclerosis, L-arginine supplementation has been shown to partially reverse impaired endothelium-dependent relaxation and platelet aggregation [22]. Although several studies indicate that supply of exogenous L-arginine is rate-limiting for the generation of NO in platelets, activated macrophages, endothelial cells and brain astrocytes, one is left with the paradox that the intracellular concentrations (0.1–4 mmol/l) of L-arginine are well above the K_m for NO synthesis [23–26].

We have reported previously [27] that chronic renal failure up-regulates L-arginine transport in human platelets via a high-affinity system y^+L, which mediates Na^+–dependent cationic and Na^+-dependent neutral amino acid transport. System y^+L amino acid transporters (y^+LAT1 and y^+LAT2) have been identified, and the association of y^+LAT (light chain) and 4F2hc (heavy chain) induces y^+L transport activity [28–30]. L-Arginine transport is also increased in RBCs from chronic renal failure patients; however, we found that up-regulation of transport is mediated via the high-capacity cationic amino acid transport system y^+ [31,32]. Since there are no reports on arterial hypertension-induced changes on L-arginine transport, we have compared L-arginine transport in RBCs and platelets in control subjects and patients with essential hypertension and in WKY (Wistar–Kyoto) rats and SHRs (spontaneously hypertensive rats). Additionally, NOS activity in platelets and plasma concentrations of L-arginine and other amino acids were investigated in normotensive and hypertensive patients.

**Table 1** Characteristics of hypertensive and normotensive groups

<table>
<thead>
<tr>
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<th>Hypertensive patients</th>
<th>Controls</th>
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<tr>
<td>n</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49 ± 2</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>30/4</td>
<td>31/5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>142.5 ± 2.4^*</td>
<td>116.2 ± 2.3</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>91.4 ± 1.5^*</td>
<td>66.3 ± 1.2</td>
</tr>
<tr>
<td>Body mass index (kg/m^2)</td>
<td>22 ± 1</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>192 ± 5</td>
<td>191 ± 14</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>109 ± 11</td>
<td>98 ± 17</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>46 ± 9</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>123 ± 7</td>
<td>102 ± 26</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>93 ± 10</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.08 ± 0.1</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>4 ± 0.2</td>
<td>4 ± 0.9</td>
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**METHODS**

**Control and hypertensive subjects**

Patients (n=34) with uncomplicated essential hypertension in stage I and 36 normotensive controls matched for age (49 ± 2 compared with 45 ± 2 years respectively) participated in the present study. Characteristics of hypertensive and normotensive groups are summarized in Table 1. The patients were on conventional treatment for hypertension (β-blockers + diuretics (n = 17), β-blockers + calcium antagonists (n = 4), ACE (angiotensin-converting enzyme)-inhibitors (n = 4) and ACE-inhibitors + calcium antagonists (n = 9)). Blood samples were drawn by venipuncture. The exclusion criteria were dyslipidaemia, recent blood transfusion and heart and renal failure. This project was approved by the Pedro Ernesto Hospital Ethical Committee, and informed consent was obtained from each of the control subjects and patients.

**SHRs**

Six normotensive (180–200 g) WKY rats and six SHRs (180–200 g) aged 20 weeks were included in the study. The rats were sedated (diazepam; 5 mg/kg of body weight, intraperitoneal) and anaesthetized with ketamine (30 mg/kg of body weight, intraperitoneal). Samples of blood from hypertensive and control rats were taken via heart puncture. All procedures were performed in accordance with the Animals Scientific Procedures Act 1986 of Rio de Janeiro, Brazil.

**L-Arginine influx in RBCs from control and hypertensive patients and SHRs**

Blood samples were centrifuged to separate the plasma. RBCs were then washed with saline [140 mmol/l NaCl,
Inhibition of system y⁺ L in hypertension

5 mmol/l KCl, 5 mmol/l d-glucose and 10 mmol/l Mops (pH 7.4). To achieve zero-trans conditions, human and rat RBCs were incubated at 37 °C for 3 h. Influx of radio-labelled l-arginine was measured as described previously and resolved into the saturable transport components y⁺ and y⁻L, by selective inhibition of system y⁺ with NEM (N-ethylmaleimide; 200 µmol/l) [31]. Aliquots (0.5 ml) of an RBC suspension in saline (haematocrit, 5–10 %) were placed in duplicate Eppendorf microcentrifuge tubes. To these, l-[³H]arginine was added at concentrations ranging from 5–500 µmol/l, and the cells were incubated at 37 °C for 5 min. After incubation, the cells were washed, centrifuged (2000 g for 10 min) and resuspended in a cold isotonic medium [107 mmol/l NaCl, 4.6 mmol/l KCl, 1.5 mmol/l CaCl₂, 1.2 mmol/l NaH₂PO₄, 1.2 mmol/l MgCl₂, 15 mmol/l NaHCO₃ and 11 mmol/l glucose (pH 7.4)], as described previously [27]. A portion (0.5 ml) of resuspended platelets was incubated at 37 °C, and l-[³H]arginine influx (1–50 µmol/l) measured over 5 min. l-Leucine (10 mmol/l), a substrate for system y⁻L, was used to resolve total l-arginine transport in platelets into system y⁻L and diffusion. Transport was terminated by rapid centrifugation, followed by two washes with Krebs buffer, re-centrifugation and lysis by Triton X-100 for β-scintillation counting. Platelets were counted using a Coulter counter.

Measurement of platelet NOS activity

NOS activity was determined from the conversion of l-[³H]arginine into l-[³H]citrulline as described previously [25], and platelets from normotensive and hypertensive subjects were studied in the absence of cofactors as described previously [33]. Platelet suspensions were incubated at 37 °C in the absence or presence of ADP (10 µmol/l) or l-lysine (1 mmol/l) for 45 min. l-[³H] Arginine (2 µCi/ml) plus unlabelled l-arginine (1 µmol/l) was added, and the incubation was continued for an additional 45 min at 37 °C. All reactions were stopped by rapid centrifugation (12 000 g for 15 s), followed by two washes with Krebs buffer. The platelet pellet was lysed with 0.1 % Triton X-100 and applied to a Dowex cation exchange resin column. l-[³H]Citrulline was eluted with 2 ml of water, and radioactivity was measured by liquid scintillation counting.

Measurement of amino acid concentrations by HPLC

As described previously [31], individual amino acids were measured by reverse-phase HPLC, using fluorescence of the orthophthalaldehyde derivatives with an automated sample processing device (Anachem, Luton, Beds., U.K.). The separation resolved all the known plasma amino acids over a 45-min analytical time period.

Chemicals

All chemicals were of the highest grade and obtained from Sigma (Poole, Dorset, U.K.).

Statistics

Data are expressed as the means ± S.E.M. of measurements in n patients or animals. Statistical significance was determined at P < 0.05 using the Mann–Whitney test. Kinetic curves for l-arginine transport were fitted with Enzfitter using a non-linear least-squares fit to the Michaelis–Menten equation.

RESULTS

L-Arginine influx into RBCs from control subjects and hypertensive patients

Total l-arginine influx was resolved in two transport systems, y⁺ and y⁻L, by the selective inhibition of system y⁺ with NEM (200 µmol/l). l-Arginine influx via system y⁻L was reduced significantly in RBCs from hypertensive patients. In contrast, Kₘ and Vₖₐₘₚ values for l-arginine transport via system y⁺ were not significantly different in RBCs from controls and hypertensive patients. Table 2 summarizes the kinetic constants calculated for l-arginine transport in RBCs from control and hypertensive subjects.

Effects of hypertension on L-arginine influx into platelets

When exogenous l-leucine (10 mmol/l) was used to isolate l-arginine transport via system y⁻L, influx of l-arginine was reduced from 86 ± 17 pmol·10⁹ cells⁻¹·min⁻¹ in controls to 36 ± 9 pmol·10⁹ cells⁻¹·min⁻¹ in platelets from hypertensive patients (P < 0.05). The Kₘ of system y⁻L for l-arginine was also significantly lower in hypertensive patients compared with controls (11 ± 3 µmol/l compared with 43 ± 7 µmol/l; P < 0.05). The diffusional component was not different between hypertensive patients (0.12 ± 0.01 h⁻¹) and controls (0.2 ± 0.04 h⁻¹).
Table 2  Kinetic parameters for L-arginine transport via systems y\(^+\) and y\(^+\)L in red blood cells from controls and hypertensive patients
Values are means ± S.E.M. of measurements from 18 controls and 16 hypertensive patients. \(^*\)P < 0.05 compared with controls.

<table>
<thead>
<tr>
<th></th>
<th>System y(^+)</th>
<th>System y(^+)L</th>
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<tr>
<td></td>
<td>(V_{\text{max}}) ((\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{cells}^{-1} \cdot \text{h}^{-1}))</td>
<td>(K_m) ((\mu\text{mol/l}))</td>
</tr>
<tr>
<td>Controls</td>
<td>275 ± 27</td>
<td>75 ± 17</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>210 ± 23</td>
<td>90 ± 17</td>
</tr>
</tbody>
</table>

Plasma amino acid profiles in controls and hypertensive patients
Blood samples were separated into plasma for analysis of amino acid levels. Plasma concentration of L-arginine was raised significantly in hypertensive patients (175 ± 19 \(\mu\text{mol/l}\)) compared with controls (137 ± 8 \(\mu\text{mol/l}\)). The plasma concentrations of L-ornithine and L-lysine were also increased in hypertensive patients (Figure 1). In contrast, L-citrulline levels in plasma were not altered by hypertension (57 ± 3 \(\mu\text{mol/l}\) compared with 49 ± 3 \(\mu\text{mol/l}\) in controls).

Comparison of L-arginine influx in RBCs from control WKY rats and SHRs
After inhibiting system y\(^+\) with NEM (200 \(\mu\text{mol/l}\)), \(K_m\) and \(V_{\text{max}}\) values determined for the remaining fraction of L-arginine influx via system y\(^+\)L were reduced significantly in RBCs from SHRs compared with WKY rats (Table 3). In contrast, kinetic constants for L-arginine transport via system y\(^+\) and diffusion were similar in SHR and WKY animals (Table 3).

DISCUSSION
Hypertension has been investigated extensively, yet its genesis and underlying mechanisms remain elusive. Hypertension can result as a consequence of a decrease in vascular lumen or an increase in plasma vascular volume. NO is a fundamental mediator in the regulation of renal...
fluid and plasma volume, cardiac function and vascular tone and, consequently, a change in the L-arginine/NO pathway can lead to hypertension [14].

Although several studies indicate that supply of exogenous L-arginine may be rate-limiting for the generation of NO in different cells one is left with the paradox that the intracellular concentrations of L-arginine are well above the $K_m$ for NO synthesis. This intriguing mechanism has been termed the ‘L-arginine paradox’, and accumulating evidence suggests that exogenous L-arginine increases the synthesis of NO [34]. The presence of increased concentrations of ADMA (asymmetric dimethylarginine) in the plasma of patients with renal and heart failure, hypercholesterolaemia and atherosclerosis could be another explanation for the L-arginine paradox [35]. In endothelial cells, the co-localization of eNOS and CAT (chloramphenicol acetyltransferase) transporters in caveolae could also help to explain the conundrum [36], although, in macrophages, for example, NOS is cytosolic. Recently, it has been suggested that, in macrophages and endothelial cells, iNOS and eNOS respectively, have access to different intracellular L-arginine pools [37].

Our present results provide the first evidence that L-arginine transport is decreased in both RBCs and platelets from hypertensive patients and is associated with an increased plasma concentration of L-arginine. The diminished rate of L-arginine transport in blood cells in hypertension is mediated selectively by a down-regulation of system y+L activity. The low capacity transport system y+L accumulates L-arginine more efficiently than system y+ [30], and over a prolonged period of time this would lead to a greater intracellular L-arginine level. The down-regulation of y+L activity observed in the present study may account for a reduced L-arginine intracellular concentration for NO production in hypertension.

The present study confirms previous observations [18] that platelets from hypertensive patients generate less NO than control platelets, with production of NO reduced by 45% in platelets from hypertensive patients compared with controls. Membrane transport of L-arginine seems to be a rate-limiting step for NO synthesis under certain conditions [23–26], and the inhibitory effects of cationic (L-lysine) amino acids on NO production provide convincing evidence that L-arginine transport is rate-limiting for the decreased rates of NO production in platelets from hypertensive patients. Impaired NO production may be related to abnormal platelet and RBC aggregability in hypertension, both of which are known to contribute to the development of vascular complications in this disease [14,18–21].

In SHRs, L-arginine influx via system y+L was also inhibited in RBCs. A previous study [38] has shown that L-arginine transport is reduced in cultured aortic smooth muscle cells isolated from SHRs compared with WKY rats. This finding, together with our present study, suggests that SHRs provide a useful model of hypertension in which alterations in L-arginine transport mimic those observed in patients with essential hypertension.

Molecular studies have recently discovered that y+LAT associates with 4F2hc to induce transport system y+L [28–30], but the mechanisms involved in its regulation have not yet been fully elucidated. Previous studies with erythrocytes and fibroblasts from patients with lysinuric protein intolerance have reported normal rates of L-lysine and L-arginine influx via system y+L [39,40]. Our present findings provide the first evidence of a disease-induced alteration of system y+L transport activity in human blood cells. Thus further studies are necessary to define the factors responsible for the inhibition of y+L activity in hypertensive patients and SHRs. There are several reports of altered RBC and platelet membrane lipid composition and fluidity in various forms of arterial hypertension [41–43]. It has been shown that a particular lipid bilayer environment is required for the activity of membrane proteins [44], and perhaps the alterations in the lipid bilayer composition in hypertension may affect membrane transporters such as system y+L.

In addition, we have found elevated levels of L-arginine in plasma taken from hypertensive patients, which may, in part, explain the controversial reports of the beneficial effects of L-arginine infusion on endothelial function observed in this pathology [14,22,45,46].

In conclusion, our results suggest that in human essential hypertension increased plasma L-arginine levels may not be sufficient to maintain NO synthesis in the presence of reduced rates of transport. The mechanisms by which system y+L is impaired in hypertension remain to be elucidated and may provide further insights into the pathophysiology and treatment of hypertension. A limitation of the present study is that hypertensive patients were on anti-hypertensive drugs. Although there are no reports of effects of anti-hypertensive medication on L-arginine transport and NO synthesis in platelets, one cannot exclude the influence of such medication. In hypertension, if L-arginine transport is a rate-limiting step for NO synthesis in platelets and RBCs, mechanisms to increase L-arginine transport could provide new therapeutic strategies to prevent hypertensive cardiovascular complications.

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