Glucose as a factor of importance for regulating arginase activity in red blood cells

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Abstract:

**Background:** Nitric oxide (NO) is an important biological messenger in the cardiovascular system. NO causes vasodilation, inhibits inflammation and platelet activation. The bioavailability of NO is reduced in various cardiovascular diseases including atherosclerosis and diabetes compared to a healthy state. NO is formed by the enzyme nitric oxide synthase (NOS) which can be found throughout the vasculature. The amino acid L-arginine is a substrate in the reaction of NO production. The mechanism of reduced bioavailability of NO is not completely understood but it is thought to be mainly reduced production of NO and increased inactivation of NO. Emerging evidence suggest that the enzyme arginase is a major contributor to this mechanism. Arginase also uses L-arginine as a substrate to produce urea and ornithine. Experimental data demonstrate that arginase activity is increased in rats with type 2 diabetes compared with healthy controls. Glucose has been reported to regulate arginase activity in endothelial cells. Red blood cells (RBCs) contain high levels of arginase which regulates NO but this regulation in RBCs and it's function in these cells is unknown. The aim of this study was to test the hypothesis if there is a difference in arginase activity in RBCs between healthy individuals and patients with type 2 diabetes. Another objective was to investigate if high glucose concentration would affect arginase activity in red blood cells.

**Methods:** Red blood cells were extracted from healthy individuals and patients with type 2 diabetes. These cells were incubated for 24 and 48 hours in a buffer solution containing 5.5 mM or 25 mM glucose. Arginase activity was determined by measuring the amount of urea produced using spectrophotometry.

**Results:** Arginase activity at baseline, (i.e. 0 hours) of incubation did not differ between healthy individuals and patients with type 2 diabetes. Arginase activity decreased with time in both groups. Arginase activity reached a steady-state at 24 hours of incubation. There was no significant difference between arginase activity whether they were incubated with 5.5 mM glucose concentration or 25 mM glucose concentration.

**Discussion:** According to the results of this study glucose does not effect arginase activity in RBCs. There is not a significant difference in arginase activity in red blood cells between healthy subjects and patients with type 2 diabetes.

**Keywords:** Nitric oxide (NO), nitric oxide synthase (NOS), arginase, arginase activity, diabetes type 2, glucose, incubation.
ABBREVIATIONS

CAD  Coronary artery disease
CRP  C-reactive protein
eNOS  Endothelial nitric oxide synthase
Hb1Ac  Glycosylated hemoglobin
HDL  High-density lipoprotein
KH  Krebs-Henseleit
LDL  Low-density lipoprotein
NO  Nitric oxide
NOS  Nitric oxide synthase
RBCs  Red blood cells
1 INTRODUCTION

1.1 Nitric Oxide

Nitric oxide (NO) is a biological signaling molecule and an important regulator of vasodilation and cardiovascular function as well as inhibition of platelet aggregation and leukocyte adhesion(1). It is also known as a neurotransmitter in the central nervous system and to play a role in the immune system/inflammation. In the vasculature NO is produced from conversion of L-arginine to L-citrulline by the endothelial isoform of the nitric oxide synthase (eNOS). This enzyme, eNOS, is constitutive, calcium-calmodulin dependent and releases picmoles of NO in response to receptor stimulation(1). Considering that NO is highly reactive and that it diffuses freely across biological membranes(2) combined with the fact that it has a short half-time makes this messenger an ideal paracrine and/or autocrine factor in the cardiovascular system(3). It's main effect on the vascular system is through regulation of vascular tone, regulation of myocardial contractility, antithrombotic effects in the vasculature, regulation of endothelial-leukocyte interactions, regulations of endothelial integrity and permeability and regulation of vascular cell proliferation(3). To further identify the effect of NO on the vascular system a competitive inhibitor of eNOS was used in an in vitro model which showed that inhibition of eNOS caused a vasoconstriction in the forearm arterial circulation in humans(1). The vasodilation tone is maintained by physical activation of endothelial cells by a stimulus such as pulsatile flow or shear stress (1). Impaired production of NO and or bioavailability is associated with a number of disease or conditions such as atherosclerosis, hypertension, diabetes, heart failure, stroke and pulmonary arterial hypertension(4).

1.2 Red blood cells and eNOS

The cardiovascular effects mediated by NO is generally considered to be derived from eNOS in endothelial cells. Red blood cells (RBCs) also contain this enzyme (5, 6). However there has been of controversy whether it is active or not(6, 7) in human RBCs. More so it has remained controversial whether or not the NO produced by RBCs can escape the scavenging of hemoglobin and be of use to the vasculature(8). It has been suggested that RBCs can export NO bioactivity and that NO from RBCs contributes to regulation of blood flow(9). Another study has demonstrated that NO generated by RBCs exhibits the same biological effects(10) as NO produced elsewhere in the vascular system.
1.3 Arginase

Arginase is a manganese metalloenzyme that hydrolyzes L-arginine, the same amino acid as eNOS, to L-ornithine and urea (11). It is one of the primary enzymes of the hepatic urea cycle and is central to the disposal of excess nitrogen coming from metabolism of amino acids and nucleotides. It exists in two isoforms, arginase I and II, sharing 60% amino acid sequence homology (12), and both forms are found throughout the body. Arginase I a cytosolic enzyme and is the one mainly located in the liver while arginase II is a mitochondrial enzyme, most abundant in kidney (13). Arginase is also believed to be of importance to the cardiovascular system. It is expressed in the vasculature (4), endothelial cells and vascular smooth muscle cells (14, 15), with species and regional variations regarding the isoform expression (4, 16, 17).

The affinity of L-arginine is higher for purified eNOS than for arginase however the maximum activity of arginase is more than 1000 times that of eNOS (18). In macrophages it has been shown that most of L-arginine was consumed in urea production rather than NO and that inhibiting arginase resulted in augmented NO production (19).

Upregulation of arginase is stimulated by a number of pro-inflammatory factors such as lipopolysaccharides, tumor necrosis factor alpha, interferon gamma (20-23). Interleukins affect arginase expression in macrophages (24). Other factors that upregulate expression of this enzyme are oxidised low-density lipoprotein (25), glucose (26), thrombin (27), hypoxia (28), angiotensin II (29), reactive oxygen and nitrogen species (30, 31) and NADPH oxidase (32).

In response to shear stress or receptor stimulation, NO is released from the vascular endothelium promoting blood flow by reducing vascular tone and platelet aggregation. Impaired NO synthesis and bioavailability is the cause of endothelial dysfunction and a central factor to many cardiovascular disorders. Reduced bioavailability of NO is one of the defining factors in endothelial dysfunction which under a broad view classifies as an imbalance between vasoconstriction and vasodilatation factors (33). Emerging evidence support the implication that arginase is a regulator of NO production and a major factor in endothelial dysfunction in many cardiovascular diseases (4). Arginase can reciprocally regulate eNOS activity thereby promoting endothelial dysfunction (34). Arginase modulates the activity of NOS by regulating the intracellular levels of L-arginine (34). Furthermore, arginase inhibition leads to the improved endothelial function as demonstrated in different animal models and humans. A recent study showed that patients with coronary artery disease (CAD) and type 2 diabetes and patients
without CAD and with type 2 diabetes had markedly improved endothelium-dependent vasodilatation following local arginase inhibition(15). This improvement or protection has been reported in another study that contained a model of ischaemia-reperfusion injury(35) further proving the point that arginase is important for the development of vascular complications of CAD. Many other studies have shown a link between endothelial dysfunction and increased arginase activity which seems mainly via interference with NO bioavailability(4).

Arginase has been suggested to reduce bioavailability of NO by several mechanisms. Competing with eNOS for the substrate L-arginine, uncoupling of NOS resulting in generation of NO scavengers and reactive oxgen species and also the repression of translation and stability of inducible NOS protein(19). Limiting the supply of L-arginine for eNOS, this reduction of substrate also seems to sensitize endothelial cells to the endogenous NOS inhibitor, asymmetric dimethyl-L-arginine which is elevated in various pathological conditions associated with endothelial dysfunction(36). It has also been suggested that limited supply of L-arginine promotes the uncoupling of eNOS causing generation of superoxide instead of NO(37). Reactive oxgen species can directly inactivate NO. Thus increased arginase activity could lead to reduced supply of the substrate L-arginine and cause uncoupling of eNOS and further depletion of NO and in turn endothelial dysfunction.

1.4 Arginase in disease

Research done on arginase and it’s role in endothelial dysfunction continues to expand. In a mouse model for atherosclerosis higher arginase activity was observed as compared with age-matched wild-type mice(38). Increasing evidence also supports the hypothesis that in pathological conditions such as myocardial ischaemia and reperfusion(39), stroke(40), heart failure(41), hypertension(42) and pulmonary arterial hypertension(43) increased arginase activity is present compared to healthy controls.

Arginase activity was reported to be increased in diabetes. A study using a rat model of type 1 diabetes showed significantly increased arginase activity in aortae and liver compared with healthy controls(26). In the same study simvastatin inhibited the upregulation of arginase activity whereas L-arginine did not. This study also demonstrated that exposure of bovine
coronary endothelial cells to high glucose significantly increased arginase activity via an effect that was inhibited by simvastatin (26). In a clinical study that included patients with type 2 diabetes the arginase activity in plasma was increased compared with healthy control subjects and it correlated positively with the degree of hyperglycemia(44).

1.5 Red blood cells and arginase

A recent study demonstrates that human RBCs express arginase I(10). A dose-dependent increase in extracellular accumulation of nitrate and nitrite following administration of an arginase inhibitor has been observed in this study. Furthermore, increase in nitrate and nitrate production was caused by the enzyme eNOS, further strengthening the hypothesis that arginase regulates bioavailability of NO. The same research also demonstrated cardioprotective effect following inhibition of arginase in RBCs in an ex vivo myocardial ischemia-reperfusion injury model. It has compared the effect of whole blood, RBCs and plasma to clarify the role of the RBCs in this experimental setting. This comparison underlined that this cardioprotective effect of arginase inhibition was dependent on RBCs. Using eNOS+/− and eNOS−/− knockout mice it demonstrated that the cardioprotective effect of arginase inhibition depends on eNOS. The data suggest that red blood cells have an important role in regulating NO production and export with functional cardiovascular effects. However much remains unknown about the regulating factors of arginase in red blood cells. The aim of this study was to identify if hyperglycemia increases arginase activity in RBCs and to determine if arginase activity is upregulated in patients with type 2 diabetes compared to healthy individuals.
2 MATERIALS AND METHODS

2.1 Study samples

Blood was collected from 7 patients with type 2 diabetes and 9 age-matched healthy individuals. Fasting venous blood was taken from a cubital vein from the subjects. Healthy control subjects were free of any medication.

2.2 Experimental protocol - Glucose incubation

Blood samples were centrifuged at 4°C, 2000 g for 15 minutes and plasma together with the white blood cells were removed. The RBCs were put on ice and moved to the cell lab. RBCs were then washed with modified Krebs-Henseleit (KH) buffer, which was sterile filtered using a 0.2 µm Millipore filter and the pH was adjusted to 7.4. 5 mM of penicillin/streptomycin (Penstrep) was added to the buffer solution. The first blood sample was washed containing KH buffer with 5.5 mM of glucose. The second sample was washed with KH buffer with 25 mM glucose. The washing was done two times and the supernatant was removed each time. A 6-well plate was prepared with KH + 5.5 mM glucose in two wells and KH + 25 mM glucose in two wells. 0.5 mL of RBCs were put into each well. The plate was then incubated at 37°C for 24 hours and 48 hours. At each time mark the samples were collected, lysed with a detergent-based lysate buffer and then stored in -80°C. A baseline sample of 0 hours was collected before any incubation with glucose. This procedure was done in a sterile environment with sterile equipment. The incubator itself was in optimal settings to imitate body temperature, 37°C and 5% CO₂.

For 2 subjects the same protocol was used with one adjustment. 50 µM L-arginine was then added to the KH buffer before it was put into the 6-well plate.

2.3 Arginase activity

Arginase activity assay has been described elsewhere(45). Briefly, human RBCs were lysed using a lysis buffer. MnCl₂ is a cofactor for arginase so lysates were treated with MnCl₂ and incubated at 56°C for 10 minutes to activate the enzyme. Samples were then allowed to cool down to room temperature and then each sample was given L-arginine, the substrate for arginase, for the positive samples and standard. Negative samples were given vehicle, missing
L-arginine, to distinguish between newly formed urea and background content. Samples were incubated at 37°C for 60 minutes. At 37°C urea is produced from L-arginine by arginase. The reaction was stopped by adding an acid solution (H$_2$SO$_4$–H$_3$PO$_4$–H$_2$O = 1:3:7). Then α-isonitrosopropiophenone (INP) was added to each sample and they were incubated at 95°C for 45 minutes. INP is a coloring agent that makes urea visible. The urea concentration was determined at 540 nm using spectrophotometry and then urea inhibitable fraction was calculated and used in the statistical analysis. The standard used was made from diluted urea stock 0.025 M with the range of concentration from 12.5 M to 0.1953 M of urea. A total protein concentration spectrophotometry measurement was also performed for all RBC samples with a standard to normalize arginase activity to that of the protein content in each of the samples.

### 2.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism. Student’s t-test, one-way analysis of variance and 2-way analysis of variance were used to analyse the data. Analysis of arginase activity comparison between time differences was assessed by 1-way analysis of variance. Comparison between the two groups, healthy controls versus individuals with diabetes type 2 was assessed by 2-way analysis of variance.

### 2.5 Ethical permits and licences

The study was performed in accordance with the Declaration of Helsinki and a written informed consent was collected from every participant. Ethical permission has been granted by the regional ethical committee of Stockholm.
3 RESULTS

3.1 Study subjects

Subject characteristics are presented in table 1 and 2. Table 1 shows basal characteristics of the two study subject groups. Two groups were age-matched. Blood tests have been performed for 6 out of 9 subjects in the healthy control group. Hb1Ac was significantly higher in the patients with type 2 diabetes group. Total cholesterol, low-density lipoprotein and low-density lipoprotein/high-density lipoprotein ratio was significantly higher in the control group.

Table 1. Study subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Healthy control group (n=6)</th>
<th>Patients with type 2 diabetes group (n=7)</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>63 ± 2†</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>148 ± 5</td>
<td>142 ± 10</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.8 ± 0.1</td>
<td>7.7 ± 1.4‡</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>36.5 ± 0.4</td>
<td>64.4 ± 6.1**</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.5 ± 0.3</td>
<td>1.3 ± 0.9‡</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.9 ± 0.4</td>
<td>3.3 ± 0.2***</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.5 ± 0.4</td>
<td>1.6 ± 0.1***</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>2.1 ± 0.3</td>
<td>1.2 ± 0.2*</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Significant differences by unpaired t test assuming both populations have the same standard deviation (SD): *P<0.05, **P<0.01, ***P<0.001 versus the control group. CRP indicates C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycosylated hemoglobin. † n = 9. ‡ n = 6.

Ongoing medication for the patients with type 2 diabetes is described in Table 2. All patients had glucose-lowering medication, only one patient was not on insulin but was on oral anti-diabetic drug. One patient was on both oral anti-diabetic drugs and insulin. None of the subjects in the control group were on any medication.
Table 2. Study subject characteristics: Medication

<table>
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<tr>
<td>Oral anti-diabetic drugs</td>
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</tr>
<tr>
<td>Insulin</td>
<td>6</td>
</tr>
<tr>
<td>ACE/ARB</td>
<td>5</td>
</tr>
<tr>
<td>Lipid lowering drugs</td>
<td>5</td>
</tr>
<tr>
<td>Antiplatelet drugs</td>
<td>4</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>4</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>3</td>
</tr>
<tr>
<td>Long acting nitrates</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are number of patients. ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

3.2 Arginase activity in red blood cells

The arginase activity in RBCs of the healthy subjects is represented in Figure 1A. Activity of arginase decreases significantly after 24 hours of incubation with KH buffer + glucose 5.5 mM glucose and 25 mM glucose. However, arginase activity did not decrease further when measured after 48 hours of incubation. Figure 1B displays the data for the arginase activity in RBCs from patients with type 2 diabetes. Also in this group arginase activity was significantly decreased after 24 hours of incubation with KH buffer + glucose 5.5 mM glucose and 25 mM glucose. The activity did not decrease further when measured at 48 hours of incubation.

There was no difference in arginase activity between RBCs collected from healthy subjects and patients with type 2 diabetes incubated with 5.5 mM glucose (Fig. 2A). The baseline activity measured at 0 hours is the same. There is no significant difference in arginase activity measurements done at 24 hours and 48 hours of incubation with 5.5 mM glucose. Figure 2B shows the results of the comparison done between the two groups incubated with 25 mM glucose. There is no significant difference between the groups in any of the measurements.
Figure 1. Arginase activity in human red blood cells after incubation in 37°C in presence of glucose (5.5 mM and 25 mM, respectively) in Krebs-Henseleit buffer. Panel A represents healthy control subjects (n = 9), whereas Panel B patients with type 2 diabetes (n = 7 for 0 hours and 24 hours, n = 6 for 48 hours). Arginase activity is expressed in urea (µmol/mg protein/min). Data are mean±SEM.
Figure 2: Arginase activity in red blood cells (RBCs) of healthy control subjects (n = 9) and patients with type 2 diabetes (n = 7 for 0 hours and 24 hours, n = 6 for 48 hours). Incubation of RBCs in presence of glucose in Krebs-Henseleit buffer (5.5 mM Panel A and 25 mM Panel B). Arginase activity is expressed in urea (µmol/mg protein/min). Data mean±SEM.
Preliminary results from two experiments using supplementation of 50 µM L-arginine to the previously described incubation conditions are summarized in Figure 3. There was no significant difference between the samples that were incubated with L-arginine compared with samples that did not contain L-arginine.

Figure 3: Arginase activity in red blood cells of healthy control subjects (n = 2) with and without supplementation of 50 µM L-arginine. Incubation of RBCs in the presence of glucose in Krebs-Henseleit buffer (5.5 mM and 25 mM). Data are mean ± SEM
4. Discussion

The main finding of the current study is that arginase activity in RBCs of patients with type 2 diabetes is not increased as compared with healthy age-matched individuals. Furthermore, glucose concentration does not affect the activity of arginase in both study groups. However the arginase activity is reduced to a certain limit during the incubation for both groups equally.

Previously it has been shown in rats and other animal models of diabetes that arginase activity is usually increased. When these rats are put on statins the arginase activity recovers to the same levels of their healthy controls(26). This could possibly be a confounding factor influencing the results of the current study. Most of the patients with diabetes were on a number of medications ranging from lipid lowering drugs such as statins to beta blockers. Whether the antidiabetic drugs have an effect needs to be answered. If it is the ongoing medication which are the factor that is leading to the decrease in arginase activity in patients with type 2 diabetes, there is not a lot that can be done since its hardly ethical to take a person with diabetes off their medication. However a recently published study has demonstrated that arginase activity in RBCs in patients with type 2 diabetes assessed at first clinical onset (i.e. when patients were off any medication) is significantly decreased compared to healthy controls(46). In this study arginase activity is significantly increased in plasma of these patients with type 2 diabetes compared to healthy controls.

Another issue that needs to be addressed is if these medication could halter the effect of glucose on the activity of arginase. Incubating the cells with another buffer might be of interest to see if that changes the activity of arginase. Of interest could be to incubate RBCs using the subject’s own plasma as a medium under the same conditions as described above. Plasma from patients with diabetes has been shown to have increased arginase activity(44). Perhaps there is a second messenger in plasma that is a cofactor needed for upregulation or downregulation of arginase. On the other hand, lipids in plasma could affect the activity of arginase. Furthermore, in that same study insulin was shown to have decreasing effect on arginase activity(44).

The arginase activity decreases with time and the reason behind that is not known. An interesting hypothesis would be to test if the presence of substrate itself is a confounder. In two pilot experiments we have tested if the addition of physiological concentration of L-arginine to the incubation buffer could change the activity of the enzyme. This preliminary data suggests that either added L-arginine has no effect and thus the intracellular storage of L-arginine is not
depleted in the red blood cells and another factor is causing the activity to decrease. Another possibility is that L-arginine cannot enter the red blood cells because the in vitro condition set in this protocol does not imitate physiologic conditions. Perhaps the concentration of L-arginine used was insufficient and a higher concentration should be tested before any conclusions are made regarding L-arginine supplementation with this protocol.

It might also be of interest to induce sheer stress while the red blood cells are being incubated to test if the regulation of arginase activity is mechanical.

There are many factors that can affect arginase activity in RBCs in their natural environment. However we wanted to start with the most simple model, excluding any confounders. Additional studies are needed to investigate the role of all these factors.

The main conclusion of the current study is that glucose does not regulate arginase activity in human RBCs. Furthermore, there was no difference in arginase activity between RBCs from patients with type 2 diabetes compared to age-matched healthy control subjects. Whether NO export from RBCs plays an important role for the development of cardiovascular complications in type 2 diabetes remains to be investigated in future studies.

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