GLUT-1 content and interaction with stomatin in red blood cells from species without vitamin C biosynthesis and their relevance for diabetes mellitus type 1

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Abstract

Ascorbic acid is commonly known as vitamin C. By definition, vitamins, with the exception of vitamin D, are substances which can not be synthesized but are essential for the organism. In this light vitamin C is special.
It is hypothesized that millions of years ago some species, like primates, guinea pigs and fruit bats, lost the ability to synthesize ascorbate from glucose due to an inactivation of an enzyme called L-gulono-γ-lactone oxidase. Since then, these species have been dependent on dietary intake of this micronutrient. Ascorbate is not only the most efficient watersoluble antioxidant, but also an important cofactor in neuro-transmitter or collagen biosynthesis. An inadequate intake of this vitamin leads to scurvy.
In 2008, a French researcher documented that all species that lost the ability to synthesize ascorbic acid express a different facilitative glucose transporter isoform (GLUT) in their erythrocytes. The expressed GLUT-1 transports not only glucose but also dehydroascorbate which is the oxidized form of vitamin C.
Was that different GLUT expression the keystone event for the evolutionary success of these species?
To examine the questions regarding the evolutionary benefit of this transporter expression, the kinetics of ascorbate and dehydroascorbate transport into erythrocytes from four different species were evaluated. Further recycling and disposal of the transported vitamin as well as a possible accumulation were observed.
The results demonstrate that there are three different transport types of dehydroascorbate. One which does not transport the vitamin at all, one whose transport of dehydroascorbate competes with glucose and one which absorbs dehydroascorbate completely independent of the extracellular glucose concentration.
After absorption, vitamin C is not recycled and is not disposed back into the extracellular fluid. Additionally, it is not stored in the cell until the erythrocyte undergoes apoptosis. The evolutionary benefit is found in an electron transfer across the erythrocyte membrane from intracellular ascorbate to the extracellular, oxidized form of vitamin C. In an energetic light, this recycling of extracellular Vitamin C is more efficient than the *de novo* synthesis of the micronutrient. Therefore, the erythrocyte acts not as a reservoir for Vitamin C storage, but as a reservoir for electron storage to prevent degradation and loss of dehydroascorbate in times of high oxidative stress. This electron reservoir becomes more important

in diseases with high levels of oxidative stress.

A metabolic disorder, which is frequently described to be accompanied by high levels of oxidative stress and lowered vitamin C levels in plasma and cells, is diabetes mellitus. The decreased plasma concentrations do not result from a smaller dietary intake. Probably, the uptake of dehydroascorbate into erythrocytes, and, therefore, the extracellular ascorbate recycling is disordered.
Investigations of the distribution of GLUT-1 in different erythrocyte membrane subdomains showed that the regulation of this transporter is altered in subjects with diabetes mellitus type 1 compared to healthy controls. *In vitro* no differences in dehydroascorbate transport rate could be observed but significantly decreased intra-erythrocyte vitamin C concentrations were detected *in vivo*.
In conclusion, the altered regulation of GLUT-1 in the erythrocyte membrane in the case of diabetes can affect vitamin C recycling in plasma. A decreased ascorbate pool in the cells leads to a decreased recycling capacity, and, therefore, to a lower antioxidant defense outside the cell. Due to that knowledge, the recommended dietary intake of vitamin C in the case of diabetes mellitus must be reconsidered to prevent further complications.

Zusammenfassung

Ascorbinsäure ist allgemein bekannt unter dem Begriff Vitamin C. Per Definition sind Vitamine, mit der Ausnahme von Vitamin D, Substanzen, die ein Organismus nicht selbst synthetisieren kann, aber die für sein Überleben essentiell sind. In diesem Fall ist Vitamin C etwas Besonderes.
Vor vielen Millionen Jahren verloren einige Arten von Lebenwesen, wie Primaten, Meerschweinchen und Flughunde, die Fähigkeit Ascorbinsäure aus Glukose zu bilden. Auslöser hierfür war eine genetische Mutation, die zu einem inaktiven Enzym namens L-Gulono-γ-Lacton Oxidase führte. Von diesem Zeitpunkt an waren genannte Arten auf die Zufuhr dieses Mikronährstoffes mit der Nahrung angewiesen.
Ascorbat ist nicht nur ein äußerst effektives wasserlößliches Antioxidanz, sondern auch ein wichtiger Kofaktor bei der Herstellung von Neurotransmittern und Kollagen. Eine unzureichende Zufuhr führt zu der typischen Mangelerscheinung von Vitamin C, genannt Skorbut.
Im Jahr 2008 konnte eine französische Forscherin zeigen, dass alle Arten, die die Fähigkeit zur Vitamin C Synthese verloren hatten, einen anderen Glukosetransporter (GLUT) in ihren Erythrozyten besaßen. Dieser Transporter, GLUT-1, transportiert nicht nur Glukose, sondern auch die oxidierte Form von Vitamin C, Dehydroascorbat.
War diese veränderte Expression einer Transporter-Isoform das Schlüsselereignis für das Überleben dieser Arten?
Um die Frage des evolutionären Vorteils dieser GLUT-1 Expression zu erörtern, wurde die Aufnahme von Ascorbat und Dehydroascorbat in Erythrozyten von vier verschiedenen Arten analysiert. Weiterführend wurden ein intrazelluläres Recycling mit anschließender Disposition sowie eine mögliche lebenslange Akkumulation von Vitamin C untersucht.
Die Ergebnisse konnten drei verschiedene Isoformen des Glukosetransporters identifizieren. Eine, die kein Vitamin C, sondern nur Glukose transportiert, in der zweiten ist eine Konkurrenz zwischen Dehydroascorbat und Glukose deutlich ersichtlich und in der dritten Form ist die Dehydroascorbat-Aufnahme nicht von Glukose beeinflussbar.
Im Anschluss an die Aufnahme wird Dehydroascorbat zwar reduziert, aber nicht wieder in die extrazelluläre Flüssigkeit entsandt. Ebenso wird es nicht bis zum Zelltod angereichert.

Der evolutionäre Vorteil der Dehydroascorbat-Aufnahme besteht in einem Elektronenfluss über die erythrozytäre Membran von intrazellulärem Ascorbat zu den extrazellulär lokalisierten oxidierten Formen des Vitamins. Dieses Recycling von extrazellulärem Vitamin C ist energetisch effizienter als die *de novo* Synthese des Vitamins. Der Erythrozyt ist daher kein Speicher für Vitamin C, sondern ein Reservoir für Elektronen, die in Zeiten erhöhten oxidativen Stresses extrazelluläres Vitamin C durch Reduktion vor der Selbstzerstörung und der Ausscheidung schützen. Dieses Reservoir für Elektronen gewinnt in Erkrankungen an Relevanz, in denen vermehrt oxidativer Stress entsteht.

Diabetes mellitus ist eine Erkrankung des Zuckerstoffwechsels, die oft mit erhöhtem oxiativen Stress und verminderten Vitamin C-Spiegeln in Plasma und Zellen einhergeht. Diese verminderten Konzentrationen resultieren hierbei nicht von einer inadäquaten Zufuhr des Mikronährstoffes mit der Nahrung. Möglicherweise ist die Aufnahme von Dehydroascorbat in die Erythrozyten gestört, so dass weniger Elektronen für das Recycling von extrazellulärem Vitamin C bereitgestellt werden können.
Untersuchungen einer Verteilung von GLUT-1 zwischen verschiedenen Membranabschnitten im Erythrozyt zeigten, dass die Regulation des Transporters bei Patienten mit Diabetes verändert ist. *In vitro* resultierten keine Unterschiede im Transportverhalten von dieser veränderten Verteilung des Transporters. *In vivo* wurden jedoch signifikant verminderte Vitamin C Konzentrationen in Erythrozyten von Personen mit Diabetes beobachtet.
Schlussendlich beeinflussen die gefunden Unterschiede in der GLUT-1 Distribution das Vitamin C Recycling im Plasma. Ein verminderter Gehalt an Vitamin C in den Erythrozyten führt zu einer schlechteren Reduktion von extratellulärem Vitamin C und damit zu einem geringeren antioxidativen Schutz. Mit diesen neuen Erkenntnissen ist es nötig die Zufuhrempfehlungen für Patienten mit Diabetes noch einmal zu überdenken, um Ihnen einen optimalen Schutz vor oxidativem Stress und damit verbundenen Folgeerkrankungen zu gewährleisten.

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AA	ascorbate
AfR	ascorbyl free radical
Ao	ascorbate oxidase
ATP	adenosine triposphate
AUC	area under the curve
BMI	body mass index
DHA	dehydroascorbate
DNA	deoxyribonucleic acid
DRM	detergent resistant membranes
EDTA	ethylenediaminetetraacetic acid
G6PDH	glucose-6-phosphate dehydrogenase
GLUT-1	facilitative glucose transporter isoform 1
GLUT-3	facilitative glucose transporter isoform 3
GLUT-4	facilitative glucose transporter isoform 4
GR	glutathione reductase
GSH	reduced glutathione
GLO	L-gulono-γ-lactone oxidase
Hb	hemoglobin
HbA$_1c$	glyco-hemoglobin A
HMEC-1	immortalized human microvascular endothelial cells
HMP	hexose monophosphate pathway
HPLC	high performance liquid chromatography
kDa	kilo Dalton

LSMEANS	least square means
NADPH+H$^+$	reduced nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
PCA	perchloric acid
PVDF	polyvinylidene difluoride
RBC	red blood cell
RDA	recommended daily allowances
RDI	reference daily intake
ROS	reactive oxygen species
RT	room temperature
SDS PAGE	sodium dodecylsulfate polyacrylamide gel eletrophoresis
TCA	trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine

Chapter 1

Introduction

1.1 Vitamin C and evolution

Ascorbic acid is commonly known as vitamin C. By definition, vitamins, with the exception of vitamin D, are substances that cannot be synthesized by, but are essential to the organism. In this light, vitamin C is special. All living species have the genome to synthesize ascorbic acid from glucose. The last step of this synthesis is the oxidation from L-gulono lactone to L-ascorbic acid by the enzyme L-gulono-γ-lactone oxidase (GLO) [1]. Some species including men, monkeys, fruit bats and guinea pigs lost this ability millions of years ago due to mutations leading to the inactivation of this enzyme [2, 3]. Henceforward, these species have been dependent on dietary vitamin C intake. Because of missing storage systems for ascorbate (AA), the plasma and extracellular concentrations of the antioxidant are seasonally fluctuating [4].

Ascorbic acid acts as co-factor in copper-dependent monooxygenases and iron-dependent dioxygenases involved in dopamine and collagene synthesis, respectively [5]. Furthermore, ascorbate is the most important antioxidant in plasma [6]. In the cell, it acts as a primary antioxidant and interacts directly with radical species [7]. In the plasma membrane, it can reduce the α -tocopheroxyl radical to α -tocopherole and protect the fatty acids of the lipid bilayer from peroxidation by chain reaction [8].

With loss of ascorbate biosynthesis, certain species apperar at first glance to have been handicaped compared to synthesizing species. The deficiency of ascorbate leads to scurvy [2, 9]. In the course of evolution, only the fittest species succeeds.

Thus, a still unsolved question is how the lack of vitamin C biosynthesis has been compensated for and how these GLO-lacking species could compete against the synthesizing ones.

In 2008, Montel-Hagen and coworkers detected a different glucose transporter isoform in red blood cell (RBC) membranes in species which lost the AA biosynthesis [10]. The expressed facilitative glucose transporter isoform 1 (GLUT-1) instead of GLUT-4 also transports dehydroascorbate (DHA) which is the oxidized form of vitamin C (see figure 1.1) [11, 1, 12]. Further, that transporter connects to a membrane protein called stomatin. The interaction of these two molecules leads to the preference of DHA transport over glucose [11]. This GLUT-1 dependend transport seems to be the only way RBCs can absorb vitamin C [13, 14]. RBCs express no active sodium dependent vitamin C transporter (SVCT), and are, therefore, not able to take up AA [15]. Even when the detailed mechanisms of DHA absorption and metabolism are not fully discovered, this GLUT-1 expression in RBCs is supposed to be the key to the explanation, why species unable to synthesize vitamin C survived in the course of evolution.

1.2 Facilitative glucose transporter isoform 1, stomatin and transport of dehydroascorbate and glucose

GLUT-1 is one of 14 known glucose transporter isoforms [16, 17]. It was first isolated and purified from erythrocyte membranes, and, therefore, it is often described as the erythrocyte glucose transporter [18]. With 5% of the total protein content, GLUT-1 has the highest occurence in RBC membranes [16]. The transporter is constitutively present in plasma membrane and therefore responsible for the basal glucose uptake [19, 20]. But not all GLUT-1 molecules in the plasma membrane are constitutively active. There are suggestions that a pool of transporter molecules is ‘masked‘ and is only activated by changes in the covalent molecule structure [21]. Further, Kumar and colleagues demonstrated that a targeting of GLUT-1 molecules into lipid rafts, also called detergent resistant membranes (DRM), activates the transporter and favors the uptake of glucose [20].

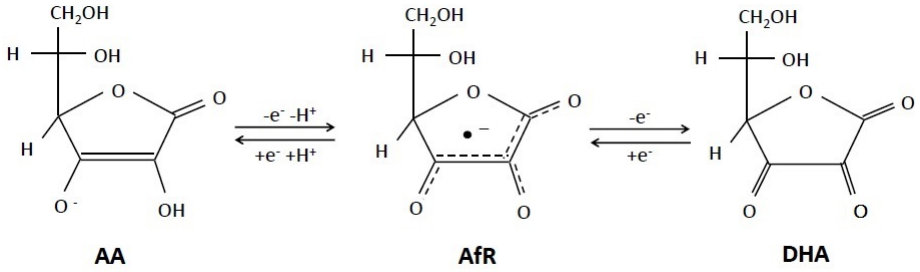

Figure 1.1: Oxidation products of ascorbate

DRM are 'free-floating' subdomains in the plasma membrane that are rich in cholesterol and sphingolipids and resistant to weak detergents like Triton X [22]. They seem to have regulative structures. Proteins which are located in these membrane microdomains are frequently modified by palmitoylation and phosphorylisation [20]. These modification of transporter proteins can affect their affinity for substrates. For example, a phosphorylisation of GLUT-1 on serine-226 or S-490 increases the transport activity of glucose [23, 24]. Further typical proteins which are found in DRM like stomatin [25] and flotillins [26] favor oligomerization of proteins which also affects the localization and activity of transporter molecules [27, 28].
Thus, an interaction of GLUT-1 and stomatin can only take place in these regulatory membrane regions. An overexpression of stomatin in cells results in decreased glucose transport into these cells [29, 30]. In summary, GLUT-1 molecules located in the plasma membrane are inactive and become activated by being modified and translocated into DRM [31]. When GLUT-1 connects to stomatin, this transporter prefers DHA as substrate [11].

1.3 Vitamin C metabolism and red blood cells

Oxidized forms of ascorbate like ascorbyl free radical (AfR) or DHA are generated by enzyme dependent oxidation or by autooxidation of this vitamin. The antioxidant can transfer up to two electrons to radical substances like reactive oxygen species (ROS) (figure 1.1 [1]). The two electron oxidized form of vitamin C, DHA, is unstable and degrades to 2,3-diketo-1-gulonic acid with a half life of 5 to 7 minutes [32, 33, 34]. This hydrolysis of DHA is irreversible and results in the urinary excretion of the former vitamin. *In vitro*, this degradation can be inhibited

by addition of reduced glutathione (GSH) [35], but the addition of GSH to plasma can not recycle AA *in vivo* [36].
In plasma, DHA concentrations of <2µM can be found [37, 32]. The missing accumulation of DHA in plasma results from the described degradation and from the fast absorption of the GLUT-1 substrate into RBCs [38]. After transport into erythrocytes, the vitamin is immediately reduced back to ascorbate [36]. The erythrocytes have two possible mechanisms to regenerate that vitamin. The required electrons can be derived from reduced nicotinamide adenine dinucleotide phosphate ($NADPH+H^+$) in an enzyme dependent reduction or from reduced glutathione (GSH) in enzyme dependent or spontaneous reduction. The GSH dependent reaction is 10-fold more effective *in vitro*, but the GSH depletion of RBCs does not result in complete termination of AA recycling [39, 40]. The produced ascorbate either cannot or can only very slowly cross the erythrocyte membrane and is trapped in the cell [41, 42] .

There are two hypothesis regarding the benefit of this DHA absorption by RBCs:
First, it is possible, that this intra-erythrocyte accumulation of vitamin C to concentrations higher than in the extracellular medium is a mechanism of vitamin C storage [43]. The absorbed DHA is reduced back to ascorbate which can not leave the cell, and the vitamin is not released back to extracellular fluids until the cell undergoes apoptosis.
On the other hand it was supposed, that the RBC can transfer electrons from intracellular molecules to extracellular substances [44]. Thus, the accumulation of AA results in an increased pool of electrons which could increase the antioxidative defense in blood [45].

1.4 Vitamin C and diabetes

Decreased vitamin C plasma concentrations [46, 47, 48, 49] as well as lowered intracellular ascorbate concentrations [50, 51] were frequently observed in patients with diabetes mellitus. These observed differences were not caused by a reduced dietary intake of the vitamin compared to the healthy population.
In context of the described DHA uptake into RBCs, these lowered vitamin C concen-

trations could be explained by an increased DHA production in diabetic metabolism. In general, the level of oxidative stress is increased in diabetes compared to healthy controls [52, 53]. Greater oxidative stress results in more production of DHA molecules which irreversibly degrade, if they are not absorbed by cells like RBCs. The transport of DHA can be affected by either the rate of intracellular reduction or by a modified or disturbed interaction of GLUT-1 and stomatin or by a combination of both.

There is evidence that the glucose-6-phosphate dehydrogenase (G6PDH) activity is decreased in diabetics [54, 55]. G6PDH catalyzes the oxidation of glucose-6-phosphate to 6-phosphogulonolactone which is the entry of the hexose monophosphate pathway (HMP). The metabolism of less glucose molecules via the HMP results in a decreased number of $NADPH+H^+$. GSH is $NADPH+H^+$ dependently reduced by the glutathione reductase (GR) which also shows a reduced activity in case of diabetes and hyperglycemia [55]. GSH can directly or enzyme dependently reduce DHA as described above.

Alterations in the protein interaction of GLUT-1 and stomatin could inhibit the preference for DHA uptake leading to a competition between glucose and DHA for GLUT-1 as is observed in RBCs as well as in other cell types [56, 57, 58]. The protein interaction can only take place in DRMs. Possibly, GLUT-1 is modified in diabetes, and, therefore, unable to translocate into these membrane domains [22].

To summarize, a decreased DHA absorption by RBCs in diabetic subjects can affect the intra- and extracellular recycling of ascorbate, resulting in a decreased DHA/AA and AfR/AA ratio [59, 47], and, therefore, increased degradation and loss of vitamin C.

1.5 Objective of this research

It was reported in 2008 that all species, which lost the ability to synthesize vitamin C, express another glucose transporter in their RBCs. This GLUT-1 expression was seen as the keystone event for evolutionary success of these species. This work should give more information about the context of GLUT-1 expression and the loss of vitamin C synthesis. Further, analyses of DHA transport into RBCs from different species should reflect that the ability to accumulate DHA leads to evolutionary success of the 'fittest' phenotype.

If this hypothesis will prove correct, the benefit of this vitamin C accumulation should be identified. So far, there are contradictory hypotheses for this vitamin C accumulation: RBCs could be seen as a possibility for vitamin C storage, or as a recycling station to protect the water soluble vitamin from degradation.
Further, this thesis will focus on GLUT-1 regulation in diabetes mellitus. Frequently lowered vitamin C plasma levels have been described previously, but no explanation for this phenomenon could be proved correct. This work provides a new hypothesis to explain the lowered vitamin C plasma concentrations observed in diabetes mellitus by focusing on the regulation of GLUT-1 in RBC membranes. If there are alterations in GLUT-1 regulation and/or in interaction with stomatin, the vitamin C metabolism could be disturbed.
In summary, this work should lead to a better understanding of vitamin C metabolism in non-synthesizing species.

Chapter 2

Materials and Methods

On the following pages, all applied methods are described in detail. The exact composition and preparation of all self-made buffers can be found in the appendix.

2.1 Ethical applications

All blood sample collections were approved by an ethical committee.

Table 2.1: Ethic committees and approvals

species	ethical committee	application number
human	Medizinische Fakultät, Universitätsklinikum Tübingen	674/2013BO2
rat	Landesuntersuchungsamt Rheinlandpfalz	23177-07/G12-1-023

Pig blood was received from animals for slaughter (StaufenFleisch®, Göppingen, Germany). Therefore, no ethical application was necessary. Wistar rats were purchased from Charles River (Sulzfeld, Germany), ODS rats from Clea (Japan). Rat blood was received from animals, that were excluded from research approaches of the department for physiology and pathophysiology at the University of Mainz. The rats were older than 6 weeks with a weight of >220g.

2.1.1 Study population

Healthy subjects were recruited at University of Hohenheim. Diabetic patients were screened and included in the doctor's office of Dr. med. Anne Schreiber (Stuttgart, Germany). Included were only male persons in the age of 18 - 40 years, type 1 diabetics were only included with a glyco-hemoglobin A (HbA_1c) <10%. Excluding criteria were high blood pressure (systole >140mm Hg and diastole >90mm Hg), excess weight (body mass index (BMI) >35 kg/m^2), increased serum cholesterol (>220 mg/dl), smoking and the intake of vitamin C supplements. Detailed data are found in the appendix (table 5.1 and figure 5.1 on page 57)

2.2 Sampling and treatment of whole blood

2.2.1 Blood sampling

Whole blood from human volunteers was sustained by puncture of the *vena cubitalis*, after cleaning the skin with a disinfectant. Whole blood was drawn into EDTA-coated S-Monovettes® (Sarstedt, Nümbrecht, Germany) and immediately mixed by swinging to prevent coagulation.
Pig blood was received from the institute of farm animal science (University of Hohenheim, Germany). Blood was received from animals, which had to be culled by a butcher. Blood was also drawn in EDTA-coated S-Monovettes® and treated like described before.
The Wistar and ODS-rats were anesthetized with Florene® 100% (AbbVie, Ludwigshafen, Germany) before they were killed by neck rupture. The blood was drawn directly from the heart into an ethylenediaminetetraacetic acid (EDTA)-coated syringe and was transferred within the next two minutes into a S-Monovette® to prevent coagulation.
For plasma vitamin C analysis 1ml of the blood sample was centrifuged for ten minutes at 1000 xg and room temperature. All centrifugation procedures with rat blood were made at 1500 xg instead of 1000 xg. Plasma was stabilized by adding the same volume of 5% perchloric acid (PCA). All samples were stored at -80°C till high performance liquid chromatography (HPLC) analysis.

2.2.2 Isolating and purification of erythrocytes

For all analysis of intracellular erythrocyte AA, the cells must be free of white blood cells and plasma. To remove plasma and buffy coat completely an Easycoll® density layer was used (Merck Millipore, Berlin, Germany). Therefore Easycoll® and phosphate buffered saline (PBS) were adjusted to a density of 1.081g/ml. 2ml of this solution were transferred on the bottom of a centrifuge tube and carefully overlayed with 8 to 10 ml of whole blood. After centrifugation for 10 minutes at 1000 xg at room temperature without brake the erythrocytes moved through the Easycoll®-layer to the bottom of the tube, overlayed by Easycoll® solution following the white buffy coat and plasma-PBS on top. Everything except the red blood cells was rejected, the erythrocytes were washed once with the same volume of PBS to remove the Easycoll® solution completely.
Rat blood was centrifuged without a density layer. The buffy coat and the upper red cell layer were rejected carefully to remove all white blood cells. To ensure that all white blood cells are removed, this washing procedure was repeated twice.
Pure erythrocytes were used for analysis of the distribution of membrane proteins in DRM as well as for the uptake kinetics of vitamin C, co-culture assays with HMEC-1 cells and for age-dependent AA assays. For DRM analysis the packed pure erythrocytes were stored at -80°C after incubation in buffer, containing different amounts of glucose, AA and ascorbate oxidase (Ao) (table 2.2.2). Cell culture experiments, uptake kinetics and age-dependent AA assays were started immediately after purification of the cells.

2.3 Uptake of AA and DHA into erythrocytes

Purified erythrocytes were adjusted with PBS, containing different amounts of glucose, 100µM AA with or without Ao, to a hematocrit of 25%. For uptake kinetics after appropriate incubation time, a sample (400µl) was taken out of the cell-buffer mixture. The sample was centrifuged (1000 xg, RT, 70 seconds, brake 7), the supernatant was removed and the cells were washed once with the same volume of pure ice cold PBS. After removing the supernatant, the packed cells (100µl) were lysed in five volumes of ice cold ultra pure Water (500µl), vortexed and immediately stabilized with the same volume of 5% PCA (600µl). All samples were stored at -80°C till HPLC analysis.

Table 2.2: Final composition of incubation buffer for analysis of erythrocyte DRM proteins

Glucose (mM)	AA (µM)	Ao (mUnits/ml)
3	0	0
3	100	0
3	100	5
5	0	0
5	100	0
5	100	5
25	0	0
25	100	0
25	100	5

Cell Counting and hemoglobin (Hb) determination

To verify an equal hematocrit value in every incubating cell-buffer suspension, the cell number of 400µl sample (i.e. 100µl packed cells) was determined. Therefore one sample (400µl) of each suspension was taken and washed as described above. The packed erythrocytes were suspended in 10ml of Casyton® and mixed by swinging upside down for 20 times. 100µl of this cell solution were transferred into 10 ml of fresh Casyton® and carefully mixed by swinging. This step was repeated a second time, the final dilution was $1{:}10^6$. The measurement was done with the cell counter system Casy® using a 60µm capillary. The cell number was calculated as mean of three measurements, in each measurement the number of cells in 200µl cell suspension was counted. Only cells, with an diameter lager than 3.8µm were considered (human). Erythrocytes from animals were smaller, the limit was adjusted to 3.3µm for rat and 3.6µm for pig erythrocytes.

Another method to adjust the intracellular AA to the cell number was to determine the Hb content of one aliquot out of each approach. Therefore the samples were treated as described but not stabilized with 5% PCA. The lysed cells were transferred to a 96-well plate (Sarstedt, NÃ¼mbrecht, Germany) and the absorbance at 546nm was measured as mean of three measurements.

2.4 AA efflux measurements

RBCs were purified and incubated in PBS with 5mM glc and either 100µM AA or 100µM AA and 5mU Ao per ml solution for 3 hours. Afterwards cells were washed twice with 4ml PBS and resuspended in PBS with 5mM glucose and 200µM reduced glutathione for stabilization of released AA by the cells. The hematocrit was adjusted to 25% during the 'loading' of the cells and during the efflux measurements. For measurement of the AA release every 30 minutes a sample of 400µl were transferred into a micro vial, centrifuged (1000 xg, 70 seconds, room temperature (RT)) and the supernatant was stabilized with the same amount of 5% PCA. The samples were stored at -80°C till HPLC analysis.

2.5 Erythrocyte separation by age

Whole blood from volunteers was withdrawn like described before. 1ml of blood was centrifuged for 3 min at 1500 xg at RT, plasma was stabilized with the same volume of 5% PCA for HPLC-analyses and stored at -80°C. The buffy coat was removed and the erythrocytes were washed once with Casyton®. 300µl of the cells were transferred to a 15ml Falcon and mixed with 10ml Casyton for measurement of whole erythrocyte intracellular AA content.
Up to three ml of whole EDTA-blood from healthy and diabetic volunteers was carefully transferred on top of an density gradient of three Easycoll® layers: 2ml of a solution with a density of 1.081g/ml was under layed by 2ml of each, an 1.092g/ml and an 1.098g/ml Easycoll® solution. The gradient was centrifuged for 30 minutes at 1000 xg and RT without brake. Afterward 4 cell layers could be seen: on top of the 1.081g/ml solution under the yellow plasma was a white ring: the buffy coat. Between 1.081g/ml and 1.092g/ml was a red ring of 5-10% of all RBCs: the youngest erythrocytes. Between the 1.092g/ml and 1.098g/ml layers was the biggest amount of Cells: the middle aged erythrocytes. On the bottom of the tube (under the 1,098g/ml solution) the oldest erythrocytes accumulated (figure 2.5).

Plasma and buffy coat were rejected, young RBCs were transferred into a 15ml Falcon tube as well as the oldest cells. The cells were washed once with Casyton®. After removal of the supernatant the amount of packed cells was estimated and the cells were resuspended in an adequate volume of Casyton® so the cell number per ml

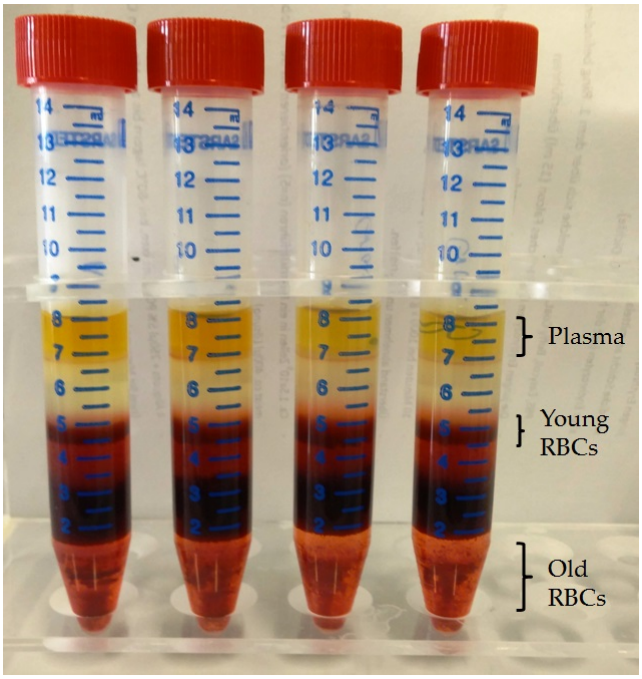

Figure 2.1: Age dependent separation of RBCs using a density layer gradient

was approximate equal in all (young, old, and unseperated) cell solutions. 5 aliquots to 400µl of each cell fraction were made. After centrifugation for 3 minutes at 1500 xg and RT the supernatant was removed and four aliquots were lysed with 250µl icecold ultra pure water and immediately stabilized with 270µl 5% PCAand stored at -80°C till HPLC-analysis. The last aliquot was lysed in 250µl ultrapure water and the Hb content was determined in three replicates at 546nm. The measured intracellular AA content was adjusted to the absorbance, so all samples could be compared to each other.

2.6 Detergent resistant membrane protein analysis

2.6.1 Isolation of erythrocyte DRM by a sucrose gradient

Purified erythrocytes were incubated in PBS with different amounts of glucose or glucose and AA respectively AA and Ao to produce DHA (table 2.2.2).
The frozen pure erythrocytes were thawed on ice. First the membranes ('ghosts') were isolated. 200µl packed cells were lysed in 15 volumes of ice cold 5mM Sørensenbuffer pH 8.0 containing 1.5mM Na_3VO_4 (phosphatase inhibitor) and

protease inhibitor cocktail (sigma-aldrich, Munich, Germany). The lysate was centrifuged at 20000 xg and 4°C for 20min to collect the membranes at the bottom of the ultracentrifuge tubes. The pellet containing the membranes and few hemoglobin molecules was precise homogenized in 5mM Sørensen buffer and centrifuged again to remove hemoglobin residues.

The pure membranes were incubated on ice for 30 minutes in PBS containing 0.5% Triton X 100, 1.5mM Na_3VO_4 and protease inhibitor (PBS-TX) to disrupted the non detergent resistant membranes. At beginning and end of the incubation time the solution was homogenized by using a 0.9mm (beginning) or a 0.6mm (end) needle to suspend the cell membranes to mechanical and physical stress. 1.5ml of this membrane homogenate were mixed with 1.5ml PBS containing 80% sucrose (table 2.3) on the bottom of an ultracentrifuge tube. This cell solution was carefully overlayed with 6ml PBS containing 30% sucrose and 3 ml PBS containing 5% sucrose. The gradient was centrifuged for 17 hours at 260000 xg at 4°C without brake.

From the top to bottom fractions of 1ml were collected and transferred into mirco tubes. In fraction 3, 4 and 5 the DRM could be seen as a white ring. The residual membrane stayed in the three fractions on the tube bottom (10, 11 and 12). Fractions 1 and 2 as well as 6 to 9 were droped.

Proteins were precipitated by 208µl 72% trichloroacetic acid (TCA) per ml sample. The samples were incubated at 4°C on a shaker for two hours followed by centrifugation (15000 xg, 5 min 4°C). The supernatant was removed and the protein pellet solved in 800µl ice cold (-20°C) acetone. All samples were frozen over night at -20°C. The next day the pellets were mechanically broken with a spatula, centrifuged and resolved in fresh acetone. This washing procedure was repeated till the pellet was crumbly. The acetone was removed with a pipette and the samples were kept on ice under the flow for 2 hours till acetone residues were evaporated. The three DRM- and the three non DRM fractions were solved in 60µl (DRM) and 80µl (non DRM) colorless SDS-buffer and stored at -20°C till protein determination and sodium dodecylsulfate polyacrylamide gel eletrophoresis (SDS PAGE).

Table 2.3: Preparation of sucrose solutions for density gradient

solution	sucrose	PBS	Na_3VO_4	protease inhibitor
80%	12g	15ml	150µl	7,5µl
30%	12g	40ml	400µl	20µl
5%	1g	20ml	200µl	10µl

2.6.2 SDS PAGE, Westernblot and immuno chemical protein detection

The protein determination was operated with the DC™ protein assay kit (Bio-Rad, Hercules, United States).
All samples were diluted to a concentration of 0.75µg protein/15µl. To 15µl Sample 5µl of 4-fold concentrated SDS buffer was added to get a final concentration of β-mercaptho ethanol of 5% and bromphenole blue of 0,03% . For all SDS PAGEs a 10% separating gel and a 5% loading gel were used. The PAGE was running for about one hour at 100V. The proteins were blotted on a polyvinylidene difluoride (PVDF)-membrane at 41mA per gel with Trans-Blot® Turbo™ transfer system (Bio-Rad, Hercules, United States).
All Antibodies were solved in TBST (tris buffered saline with 0,05% Tween 20) with 5% bovine serum albumin (CarlRoth AG, Karlsruhe, Germany). The incubating times of each solution is shown in table 2.4. After incubation with primary and secondary antibodies, the membrane was washed three times for 5 minutes with TBST to remove all residues of antibody solution. The detection of the protein bands was operated with the ChemiDoc XRS™ system by using the application 'chemi high sens'. To take a picture of the molecular weight marker the application 'epi white' was choosen. Both pictures were merged to check if the protein bands have the right molecular weight.

Densitometric evaluation of Westernblots

For statistical analysis the effects, seen of Westernblots, were brought into metric data. Therefore the blot pictures were analyzed with imageJ 1.48v (National institute of Health, United States). The blackness of the bands (pixel density) was estimated in metric data for analysis.

Table 2.4: Antibody dilutions and incubation times

target protein	description	manufactorer	dilution	incubation time
anti-stomatin	rabbit polyclonal, epitop: H-45	santacruz	1:750	overnight, 4°C
anti-GLUT-1	rabbit polyclonal, c-terminal	Merck	1:750	1 hour, RT
anti-rabbit	goat polyclonal, peroxidase conjugated	Merck	1:2000	1 hour, RT

Problems of this calculation was that only bands of the same blot could be compared to each other. Further the blackness of bands was dependent to the handling, e.g. the volume of the chemiluminescent substrate that was distributed on each blot. So on each blot all to compared samples were applicated and in the statistical analysis an effect of each blot was modeled.

2.7 High performance liquid chromatography analysis

HPLC analysis was done by using a reversed phase C18-AQ column (Dr. Maisch GmbH, Ammerbuch, Germany) with a diameter of 5µm. For mobile phase a 3.7mM NaH_2PO_4*H_2O solution was adjusted to pH 3.0 with meta-phosphoric acid. The flow rate during measurement was 1.0ml/min. A coulometric cell (Analytic cell 5011A from esa) was used, the sensivity of the detector (range) was 20µA, the potential was -300mV in the first cell, and 300mV in the second. For detection a Coulochem II detector from esa was used in combination with an autosampler and software system from Shimadzu Corporation (Duisburg, Germany).
All samples stored at -80°C were thawed at 4°C and immediately centrifuged (15000 xg, 5 min, 4°C) when thawed. All samples were kept on ice to reduce deprivation of vitamin C by temperature. Plasma samples were measured twice, once by adding 10µl pure water and a second time by adding 10µl of a 750mM Tris(2-carboxyethyl)phosphine (TCEP). TCEP is a reducing agent that reduces the oxidized forms of vitamin C (monohydroascorbate and DHA) to AA. In cell lysates

and standards always TCEP was added.
Standards were prepared fresh each time before measurement. The concentrations were 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100µM. Injections volume was 20µl for standards and samples. Analyses and Integration of the area under the curve (AUC) was calculated with LabSolutions© software 5.54 SP2 (Shimadzu Corporation, Duisburg, Germany).

2.8 Cell culture

HMEC-1 cells were received from Center for Disease Control and Prevention (CDC, Atlanta, United States). The cells were cultured in MCDB 131[2] medium (Biochrom, Berlin, Germany) with 1g/L glucose, 10% FCS (Biochrom, Berlin, Germany), trace elements (Biochrom, Berlin, Germany), 1% Penicilin- Streptomycin solution (100U/ml final concentration (Biochrom, Berlin, Germany)), Putrescin (Biochrom, Berlin, Germany), 1µg/ml Hydrocortison (sigma-aldrich, Munich, Germany), 10ng/ml EGF (Biochrom, Berlin, Germany) and 1ng/ml VEGF-α (Invitrogen, Carlsbad, United States), with 100µM AA once a week.
Cells were seeded with a concentration of $5*10^5$ cells / 75cm^2 flask. The cells were splitted once a week. Therefore the medium was removed and the cell layer was washed twice with 5ml PBS$^-$. After removing the buffer completely 1.5ml of a 0.05% EDTA - 0.02% Trypsin solution was given on the bottom of the flask and were equally distributed by swinging the flask from side to side. Then the cells were incubated at 37°C with 5% CO_2 for 4 minutes to break off the cell adhesion molecules. The cells were chipped off the flask by knocking on the bottom. When the cells were detached 10ml of medium was added to stop the enzyme activity. The cell solution was transferred into a 50ml falcon and the cells were isolated by pipetting them 15 times up and down with a glass pipette. 100µl of this homogenate were diluted in 10ml of Casyton® and the cell number per ml solution was determinate with the cell counting system CASY®. Cells were then seeded with a concentration of $5*10^5$ cells per T75 flask or 10cm dish or $2*10^5$ cells per 60mm dish. Medium was changed each day after splitting the cells.

[2]special cell culture medium for human microvascular endothelial cell lines

2.8.1 Uptake kinetics of ascorbate and dehydroascorbate into HMEC-1

For each time point of the kinetic one 10cm dish was seeded five or six days before as described above. Medium was removed and the cells were washed once with 4ml PBS^-. Fresh complete medium was prepared with 100µM AA for AA uptake, or with 5mU/ml Ao for DHA uptake. 8ml of the appropriate medium was transferred to each dish. For uptake kinetics of DHA AA was given to each dish immediately before the time was started, otherwise the enzyme began to metabolize AA to early. All dishes except the start value were stored in the incubator (37°C with 5%CO_2). The start value was lysed directly after the PBS^- was removed. Therefore 300µl 5% PCA was dripped on the cell layer and the cells were scratched off with a spatula, transferred into an microtube and homogenated by passing through a 0.9mm needle for 20 times. The cell samples were stored at -80°C till HPLC analysis and deoxyribonucleic acid (DNA) determination. The incubated cells were washed twice with PBS^- before the cells were lysed and scratched off like described before.

2.8.2 DNA determination assay

The DNA content of each cell sample was determined to relate the measured intracellular AA concentration to the cell number which was grown on a dish. Cell lysates were thawed on ice or in the fridge (4°C). 200µl homogenized cell lysate were taken out off each aliquot and centrifuged at 21000 xg for 5min at 4°C. The supernatant was transferred into an HPLC vial as described before. The pellet (proteins and DNA) was washed twice with icecold pure ethanol to remove PCA residues. Herring sperm was used for standards with concentrations of 0, 1, 2, 5, 10, 20 and 30µg DNA. All samples and standards were dried over night in a speed-vac or freeze drier. To the dried pellets were 400µl solution A' were added (16%PCA with 0,04%acetaldehyde), to the standards 200µl solution A'. Afterwards 640µl of solution B (4% Diphenylamine in pure acetic acid) was given to all samples (320µl to each standard). The samples were vortexed for 10 seconds and incubated at 37°C for about 20 hours.

After centrifugation for 30 seconds at 5000 xg each well was filled with 200µl sample or standard solution. The absorbance was measured at 590nm with reference at 750nm.

The quantitative determination occurred by the straight calibration line estimated with the standards.

2.9 Statistical analysis

All data were analyzed with statistic software SAS 9.4 (SAS Institute Inc., Cary, United States). First all data was tested for Gaussian distribution and homogeneity of variance. If necessary data were transformed before analysis. Data were analyzed by adapting a mixed model including all qualitative and quantitative parameters; persons were defined as random parameter. Degrees of freedom were adjusted after Kenward Rodger. For all analyses a p-value of <0.05 was considered to be significant and is markes with a $*$.
For each experiment a new model was adjusted to the data. For a better understanding of modeling the SAS code of uptake kinetic analysis is explained.

```
proc mixed data=up plots=studentpanel lognote;

class group number glc treat t;

model TAA= group|treat|glc group*treat*glc*time initasc
/ddfm=kr outp=resid residual solution;

random t/sub= group*number type =AR(1);
repeated t/sub= group*number*glc*treat type =AR(1);

run;
```

Figure 2.2: SAS code for the mixed model adapted for analysis of uptake kinetics in different species

The data showed no homogeneity of variances so all data were transformed by natural logarithm (TAA). In the class statement all qualitative variables were defined: 'group' for the species, 'number' for each individual, 'glc' for glucose level, 'treat' for the treatment, i.e. addition of Ao and 't' as qualitative time for calculation of lsmeans (figure 2.2). The model is composed of the fix effects group, glc and treat with all possible interactions. Further 'initasc' is taken into the model as covariable, caused by the observation, that species with a higher RBC AA to the beginning, took up more than RBCs with lower AA levels. Further the statement

'group*glc*treat*time' models a linear gradient to test of the uptake rates are different from each other and significant. The statement '\random t\sub=group*number' models an random effect for each individuum in each group in consequence if the assumption, that all samples derived from one individual are more similar to each other than samples from different individuals. For the same reason the '\repeated t\sub= group*number*glc*treat' statement was modeled with the difference that in this statement the repeated measurements over time in each treatment are focused.

For the visualization of the data all results calculated by least square means (LSMEANS) statement were transformed back. The standard errors could not be transformed back (statistically not acceptable), so all graphics with re-transformed data show no standard errors. For the original graphics of the transformed date with standard errors have a look in the appendix.

2.10 Evaluation of AA stability in PBS and turnover to DHA by ascorbate oxidase

Before start of the uptake kinetic measurements the stability of AA in PBS and the turnover from L-AA to L-DHA (the substrate for GLUT-1) by ascorbate oxidase (Ao) was controlled to guarantee physiologic concentrations. Figure 2.3 shows no changes in AA concentration in PBS with different glucose concentrations over a period of 3 hours (filled bars). Ao oxidizes AA into DHA which is not stable in PBS and thus the measured concentration continuously decreases (pattern bars). Per minute and ml 5nMol AA are converted to DHA at pH 5.6 (manufactorer's information). Figure 2.3 shows a decrease in AA concentration of 0.5µM per minute (90µM in 180 minutes) what corresponds to a conversion of 0.5nMol per minute and ml. The difference between the manufactorer's specifications and the real measured AA conversion can be explained by the difference of pH. The incubation media (pH 7.3 to 7.4) differ a lot from the enzyme's optimum pH of 5.6, so the activity of the Ao is smaller and less AA is oxidized.
In summary, the concentration of generated DHA equates to the physiological DHA concentration in plasma which is $<$2µM [37, 32].

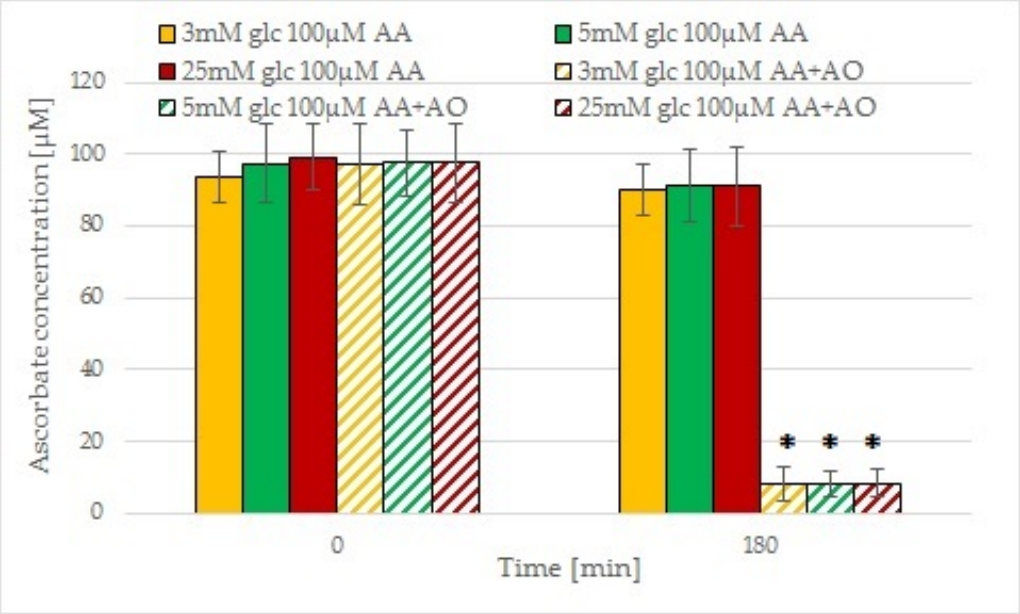

Figure 2.3: Stability of AA in PBS with and without Ao over a period of 3 hours. The initial AA content in PBS did not differ from that after 3 hours. When incubated with Ao the AA content significantly decreases over time (data denoted with $*$).

Chapter 3

Results and Discussion

The following chapter contains two thematic sections. First part focuses on the transport of AA and DHA into red blood cells (RBCs) and the scientific relevance of this uptake for species able and unable to synthesize vitamin C. The second part is about the molecular regulation of DHA transport. The distribution of the membrane proteins GLUT-1 and stomatin was evaluated to identify possible differences in RBC membrane composition in subjects with diabetes mellitus type 1 and healthy controls.

3.1 Physiologic relevance of dehydroascorbate uptake into human red blood cells

The objective of this research was to identify the evolutionary and scientific role of DHA uptake into RBCs *in vitro*. First, the uptake of AA and DHA into RBCs of different species was observed. The purpose was to examine whether only species which can not synthesize AA express GLUT-1 in their RBC membrane, and, therefore, can transport DHA. Further, a possible competition between DHA and glucose uptake by GLUT-1 was examined.

3.1.1 Ascorbate and dehydroascorbate uptake into red blood cells of different species

Erythrocytes obtained from human volunteers only absorbed the oxidized form of vitamin C (figure 3.1, page 22). In this context a significant constant transport of

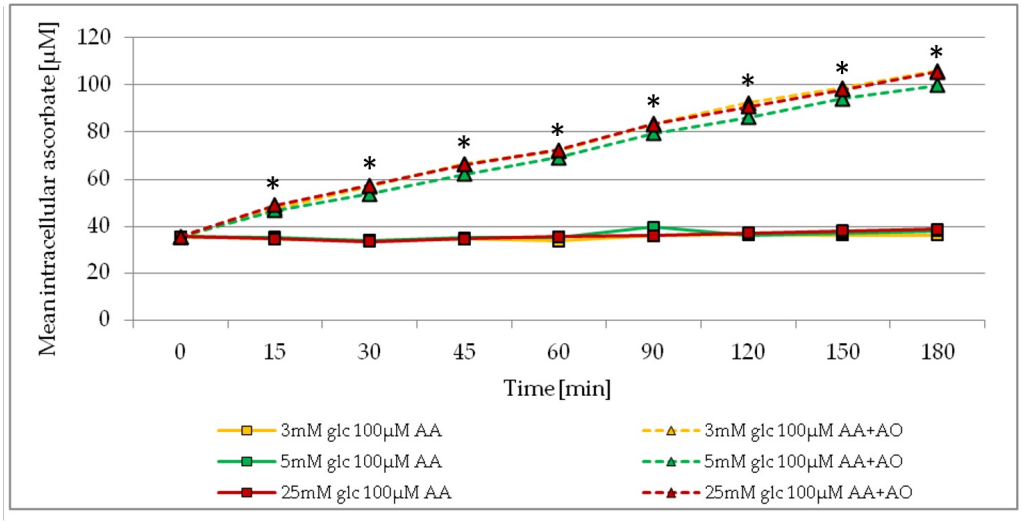

Figure 3.1: Accumulation of AA (continous lines) and DHA (broken lines) in human RBCs over a period of 3 hours (n=6). $*$ denote significant differences ($p<0.05$) in vitamin C treatment (AA compared to AA+AO) at the same glucose concentration and time. The transformed data with errorbars is shown in the appendix, fig. 5.7, page 61.

DHA over a period of 3 hours could be seen as documented before by Vera et al. [60]. The extracellular glucose concentration did not affect the transport of neither AA nor DHA.

To make sure that this uptake of DHA is specific for species which are unable to synthesize AA the absorption of AA and DHA into RBCs obtained from pigs who can synthesize vitamin C was evaluated. Pig erythrocytes took up neither AA nor DHA (figure 3.2, page 23). Therefore, it seems likely that DHA uptake into erythrocytes is an exclusive skill of species without AA biosynthesis like supposed by Montel-Hagen before [11, 10].

To ensure that the expression of GLUT-1 molecules in erythrocyte membrane was an evolutionary development, and, therefore, was not triggered by modifications during erythropoesis which are caused by the lack of AA synthesis, i.e. changes in intra- and extracellular AA concentrations in bone marrow or other tissues, the transport of DHA and AA into two rat species, Wistar rats and ODS rats, was examined.

Wistar rats are a AA synthesizing species like pigs and no transport of DHA into rat erythrocytes was documented in Spraque Dawley rats before [61]. Further, no GLUT-1 was detected in rat RBCs [11] (a representative Westernblot is shown

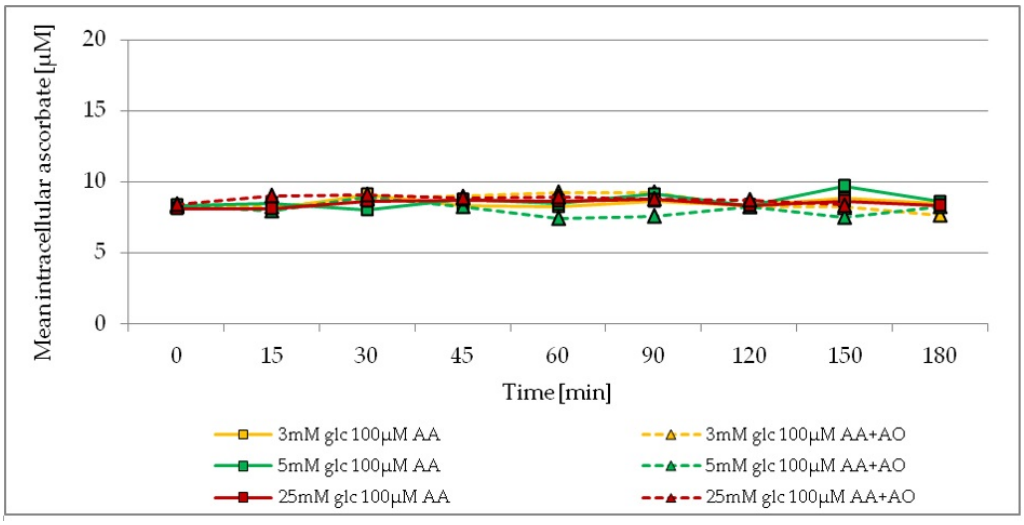

Figure 3.2: Accumulation of AA (continous lines) and DHA (broken lines) in pig RBCs over a period of 3 hours (n=4). The transformed data with errorbars is shown in the appendix, fig. 5.8, page 62.

in the appendix, fig. 5.4, page 60). Surprisingly the Wistar rats significantly absorbed DHA and accumulated the vitamin in their erythrocytes (figure 3.3, page 24). The transport was negatively affected by the extracellular glucose concentration. That means, there was a competition between glucose and DHA for the GLUT-transporter in Wistar rat RBCs. These findings indicate that Wistar rats have facilitative glucose transporter isoform 3 (GLUT-3) in their RBC membrane which is the second DHA transporting glucose transporter isoform [12, 62]. Westernblot analysis confirmed this assumption (see fig 5.5 on page 60).

In conclusion, the classification of species into those which can synthesize vitamin C and which lost this ability gives no information about whether DHA is absorbed by their RBCs. Thus, not all species with AA synthesis express facilitative glucose transporter isoform 4 (GLUT-4) in RBCs as hypothesized by Montel-Hagen previously [11, 10], and, therefore, are unable to absorb the oxidized form of vitamin C.

To identify whether the loss of an active GLO modifies the DHA transport in the same species, erythrocytes from ODS rats were isolated. ODS rats are the same species as Wistars, but lost the ability to synthesize AA due to an inactive GLO [63].

RBCs obtained from ODS rats also absorb DHA (figure 3.4, page 25). The transport

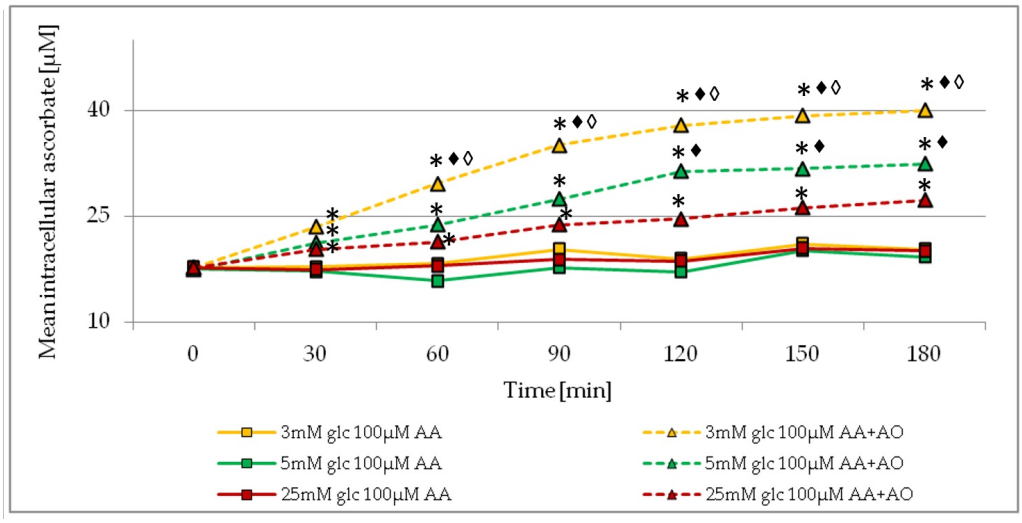

Figure 3.3: Accumulation of AA (continous lines) and DHA (broken lines) in Wistar RBCs over a period of 3 hours (n=3). $*$ denote significant differences ($p<0.05$) in vitamin C treatment (AA compared to AA+AO) at the same glucose concentration and time. $\Diamond$ marked data points are significantly different ($p<0.05$) compared to 5mM glucose but the same vitamin C treatment, $\blacklozenge$ marked data is significantly different ($p<0.05$) compared to 25mM glucose within the same vitamin C treatment. The transformed data with errorbars are shown in the appendix, fig. 5.9, page 62.

competes with the extracellular glucose concentration as seen before in Wistar rats, but the accumulated vitamin C concentration is significantly increased in ODS compared to Wistar rats (figure 3.5, page 25). Consequently, DHA transport that is independent of the extracellular glucose concentration, seems to be a human characteristic and not a universal feature of all species unable to synthesize AA.
These results indicate that RBCs from ODS rats adapted to the lacking AA biosynthesis, but not in the same way as humans, i.e. primates did. The missing competition between glucose and DHA seems to be a unique feature and cannot be universally transferred to all non-vitamin C-synthesizing species.

To identify whether this glucose-concentration-independent DHA transport can be seen in other human cell types, the uptake of AA and DHA into immortalized human microvascular endothelial cells (HMEC-1) was examined. Endothelial cells are in contact with RBCs and can also transport DHA [64]. Surprisingly the DHA uptake into HMEC-1 competes with the extracellular glucose concentration. In hyperglycemic (25mM) medium the cells take up significantly less DHA than in

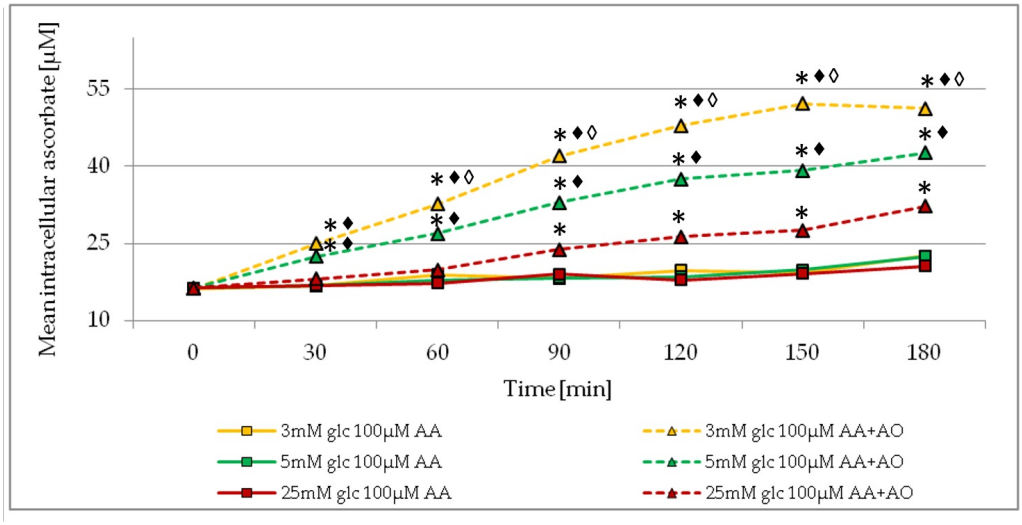

Figure 3.4: Accumulation of AA (continous lines) and DHA (broken lines) in ODS rat RBCs over a period of 3 hours (n=3). $*$ marked data points show significant differences ($p<0.05$) in vitamin C treatment (AA compared to AA+AO) at the same glucose concentration. $\Diamond$ marked data points are significantly different ($p<0.05$) compared to 5mM glucose and the same vitamin C treatment, $\blacklozenge$ marked data is significantly different ($p<0.05$) compared to 25mM glucose within the same vitamin C treatment. The transformed data with errorbars are shown in the appendix, fig. 5.10, page 63.

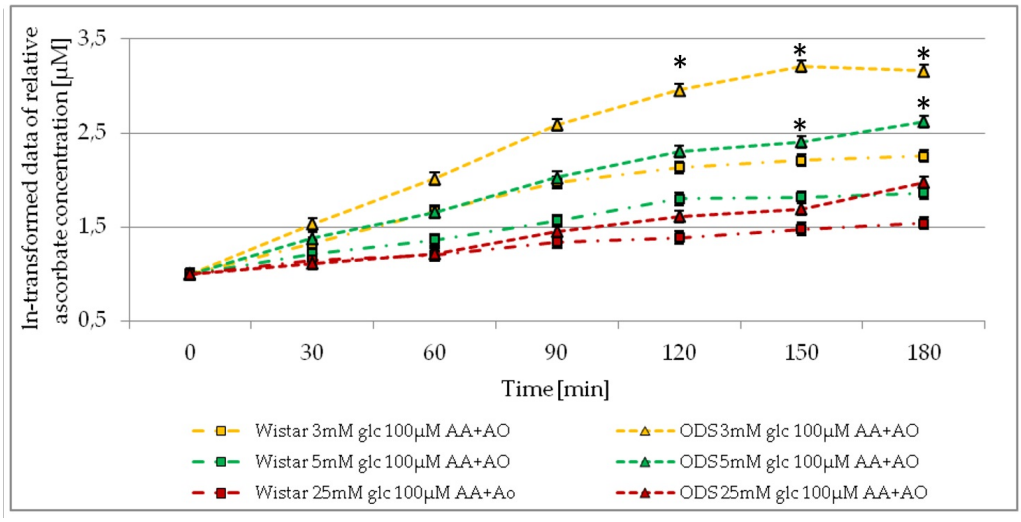

Figure 3.5: Comparison of ln-transformed data of DHA accumulation in RBCs which were obtained from Wistar and ODS rats. $*$ denotes significant differences ($p<0.05$) between the rat species at the same time and treatment.

normoglycemic (5mM) medium (Appendix, figure 5.3, page 59). This competition had been described in other human cell lines before [57, 58].

In conclusion, all these results demonstrate that a DHA transport system that is independent of the extracellular glucose concentration, is a unique characteristic of human RBCs. Neither human endothelial cells nor erythrocytes of ODS rats transport DHA without competition with the extracellular glucose concentration. But ODS rats have an increased DHA transport compared to their synthesizing relatives. Therefore, it is possible that the lack of AA biosynthesis can be compensated by modifications of the given transport molecules, resulting in an increased DHA transport rate as observed in ODS rats. But the complete glucose-concentration-independent absorption of DHA is not only a result of modifications of transport molecules. In RBCs from all analyzed species, stomatin was detected (Appendix, fig. 5.6, page 61). Therefore, stomatin cannot be the keyprotein for DHA transport. Thus, it may be presumed that the key to DHA transport, which does not compete with glucose, is the expression of GLUT-1 molecules in human RBCs.
GLUT-3 transports DHA at the same rate as GLUT-1 does, but GLUT-3 can not translocate into detergent resistant membranes (DRM) [20], and, therefore, GLUT-1 is the only glucose transporter which can interact with stomatin [29, 11]. If this interaction is the key to glucose-concentration-independent DHA absorption, the decreased transport into RBCs of ODS rats would be explained.
But still there is no explanation for the competition between glucose and DHA for transport in HMEC-1. Both cell types contain GLUT-1 and stomatin molecules in their membranes and DRM (data not shown). The only difference is that HMEC-GLUT-1 molecules have a molecular weight of $\sim$55 kDa and RBC-GLUT-1 molecules show a high variance in their weight (45 kDA to 65 kDa) (figure 3.6, page 27). These differences in molecular weight are caused by modifications of the protein like N-glycolysation [31, 23], palmitoylation [20] and phosphorylisation [24] at various sites. Probably, these post translational modifications enable the interaction of GLUT-1 with stomatin in RBCs resulting in the missing competition in transport of DHA with glucose.

But still, the question regarding the benefit of transport and accumulation of DHA in RBCs remains. Possibly, RBCs act as a kind of AA reservoir which absorbs DHA, recycles the vitamin [43, 36] and disposes AA molecules into plasma at times

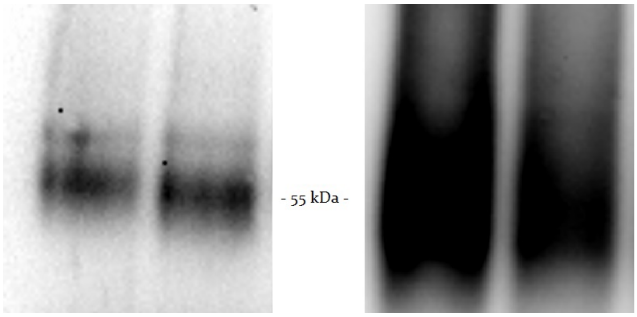

Figure 3.6: Representative Westernblot of GLUT-1 in human endothelial cells (HMEC-1, left side) and human RBC (right side).

when the extracellular level are decreasing as postulated by Hornig *et al.* [42]. A higher transport rate would lead to more reduced AA in plasma and, therefore, to a better antioxidative defense.

3.1.2 Ascorbate disposal from human red blood cells

To evaluate whether erythrocytes accumulate and reduce DHA and further release AA to the extracellular fluids, AA loaded and unloaded erythrocytes were transferred into medium without AA and the extracellular concentration of the vitamin was measured during 3 hours. Loaded RBCs released significantly more AA than unloaded cells (figure 3.7, page 28). The initial intracellular AA concentration had a significant effect on the disposed amount, i.e. the higher the intracellular vitamin C concentration at start of the measurement the more molecules were disposed of. But despite a big difference in concentration of vitamin C from ∼70µM AA inside the RBC to 0µM outside the cell the release is negligible (about 1.3µM in 3 hours in loaded and 0.5µM in 3 hours in unloaded cells). A comparable small efflux of vitamin C was documented in RBCs of guinea pigs [42]. So neither men nor guinea pigs actively disposed of reduced vitamin C into the extracellular fluids.
As there is no active transport of AA out of the cell, DHA transport via GLUT-1 could be a possible explanation for the observed results. Intracellular AA can act as an electron donor resulting in a low percentage of intracellular DHA. Also, RBCs are able to transfer electrons across their membrane to reduce extracellular substances like ferricyanide [45]. Thus a turnover from intracellular AA to DHA is likely and the produced DHA molecules can cross the membrane via facilitative diffusion by GLUT-1. Thus, the observed disposal of vitamin C is a passive efflux

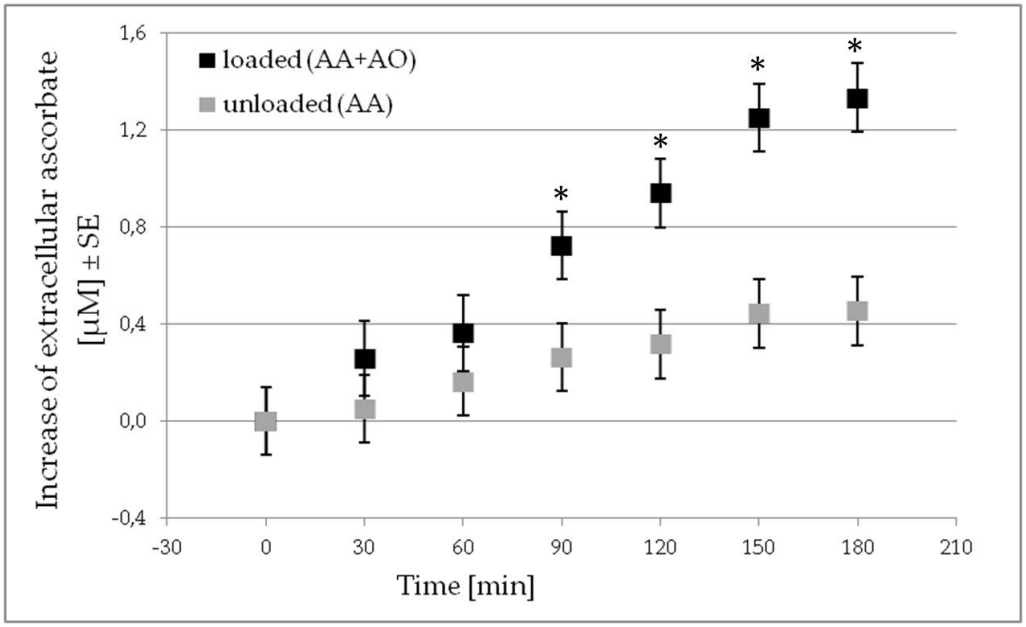

Figure 3.7: AA concentration in extracellular media where loaded and unloaded RBCs were incubated in (n=3) corrected to the initial vitamin C concentration. The increase of extracellular vitamin C is significantly higher in the medium with loaded RBCs. Significant differences ($p<0.05$) in extracellular vitamin C concentration are denoted by $*$.

of DHA. In conclusion, RBCs do not absorb and recycle DHA for AA disposal to guarantee a constant amount of reduced vitamin C in plasma.

3.1.3 Ascorbate content of different aged red blood cells

RBCs do not release AA into the extracellular fluid by active transport as demonstrated before but still the question remains why the cells absorb and recycle DHA. Since RBCs are not able to dispose of AA an accumulation of vitamin C over the whole lifespan of the cells must occur. The AA pool which is 'stored' in RBCs will be released into the circulation when the cells undergo apoptosis. This might explain, why scurvy typically occurs after vitamin C deficiency for longer than 10 weeks [65]. That precisely corresponds with the lifetime of erythrocytes [66].

Figure 3.8 on page 29 shows, that old RBCs have significantly less intracellular AA compared to middle aged and young cells. Consequently, RBC are no reservoir for vitamin C storage for times in which vitamin C is not available in the diet. The transport of DHA into RBCs diminishes over a period of 16 days *in vitro*

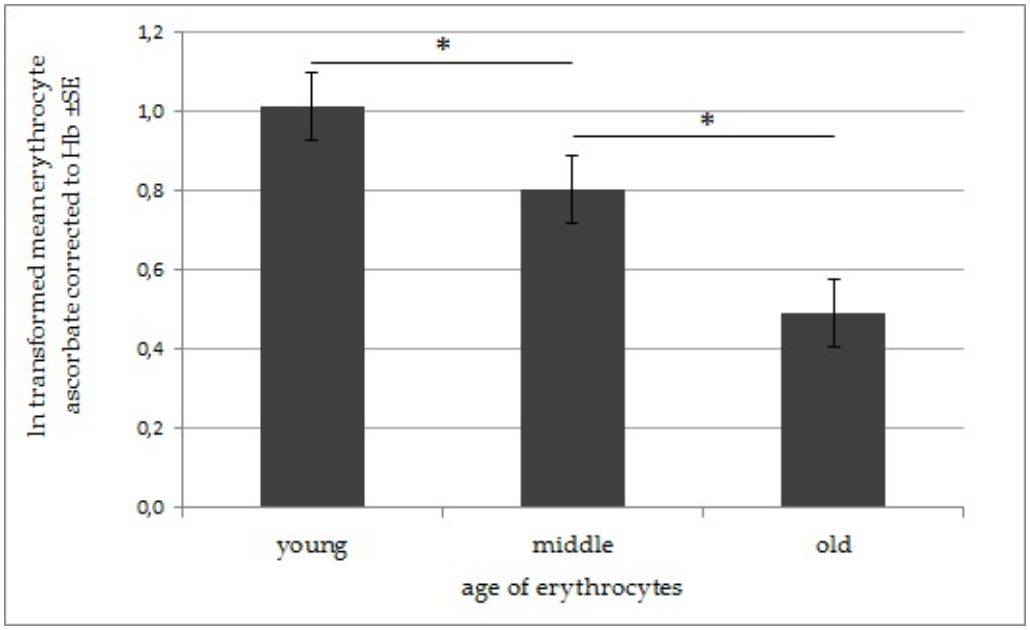

Figure 3.8: ln-transformed data of AA concentration in RBCs with different ages corrected to Hb content (n=6). The vitamin C content decreases with age. Significant differences ($p<0.05$) are denoted by $*$.

[67]. Possibly, *in vivo* the transport activity also decreases with the age of the erythrocytes and less DHA is absorbed. But without a gate to exit the cells, the intracellular vitamin C concentration must increase despite lower transport activity. There is no explanation for, where the vitamin goes or how it is used.
What is the benefit of uptake and reduction of this essential substance? And why is this transport of DHA documented in species which lost the GLO activity like humans and guinea pigs [42, 61, 56, 11], but not in any synthesizing species?

Possibly, it is not the vitamin's physiological function as a co-factor for several enzymes or antioxidant that the cells are interested in, but the molecule's chemical structure. Ascorbic acid can emit two electrons which means, one molecule of AA can quench two radicals. Further, the oxidized vitamin, i.e. AfR and DHA molecules, can be regenerated immediately because all enzymes and electron donors needed for AA recycling are available in RBCs. So, erythrocytes might not be a reservoir for vitamin C but for electron storage. James May demonstrated, that RBCs can transport electrons over their membrane to reduce extracellular molecules [45]. Further, it was shown that erythrocytes reduce extracellular AfR

molecules by a transfer of electrons from intracellular AA molecules across the membrane [68] and that RBCs are able to regenerate 35µM AA in blood every 3 minutes [36].
This reduction capacity of RBCs does not decrease immediately after inhibition of glucose and DHA transport by Cytochalasin B [7] - an GLUT-1 inhibitor [69]. Thus, the electron transfer can be sustained even when the extracellular conditions change. That might be evidence for the existence of an electron pool inside the erythrocyte.

This leads to the assumption, that the evolutionary key advantage of the expression of GLUT-1 instead of GLUT-4 in red blood cell membrane is the transport of a molecule
a) that is able to 'store' two electrons and increase the antioxidant defense pool of the organism,
b) that is much smaller than the usual electron donors $NADPH+H^+$ and GSH,
c) that can easily be recycled within the cell,
d) that can not be synthesized by the organism but is needed for many metabolic steps further and is released back to plasma when the cells undergo apoptosis and
e) that would otherwise be degraded and lost via the kidney.

In times without evolutionary pressure many different phenotypes of one species develop. Regarding the RBCs, there must had been species expressing GLUT-1, GLUT-3, GLUT-4 and other isoforms in their RBCs under the condition that these changes in transport molecules had not been a disadvantage of glucose transport.

After the loss of the L-gulono-γ-lactone oxidase (GLO) and the ascorbate synthesis, the fittest phenotypes succeeded. Figure 3.9 on page 31 shows three different pathways of glucose metabolism in a cell (under the hypothesis, that all cells have all enzymes and are able to synthesize vitamin C). Anaerobic glycolysis is the most common pathway where 6 adenosine triposphate (ATP) and no reduction equivalents are generated.
In the pathway of ascorbate synthesis, 1 glucose molecule is required to undergo glycolysis to supply the required activation energy (2 ATP). Therefore 3 glucose molecules are required for a biosynthesis of 2 AA molecules. These 2 AA molecules can transfer 4 electrons over the membrane and reduce 4 AfR or 2 DHA molecules

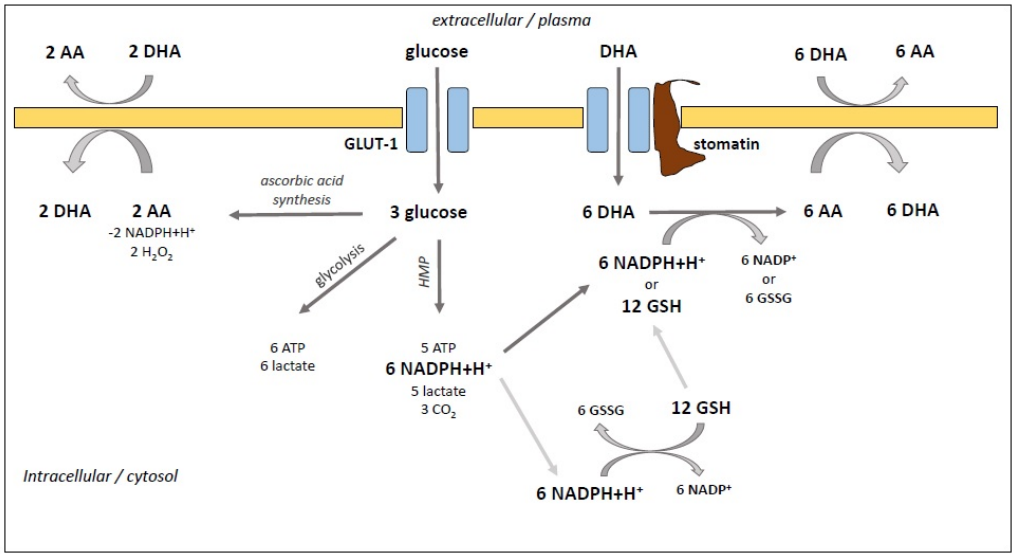

Figure 3.9: Hypotetic possibilities of intracellular glucose metabolism and its relevance for AA recycling.

in plasma. Considering the loss of 2 NADPH+H^+, even one additional glucose molecule is required to undergo the HMP. Thus, AA synthesis is from an energy counting perspective not very profitable.

In the hexose monophosphate pathway 3 glucose molecules are converted to 5 ATP and 6 NADPH+H^+. These reduction equivalents can reduce 6 intracellular DHA molecules to AA resulting in a net transfer of 12 electrons across the membrane. This enables the extracellular reduction of 12 AfR or 6 DHA molecules under the energetic loss of 1 ATP.

In an energetic light, the recycling of DHA is more efficient than the *de novo* synthesis of AA from glucose. Necessary for this recycling is the availability of vitamin C from the diet. If vitamin C is available, phenotypes expressing GLUT-3 or GLUT-1 are not disadvantaged and can compete with the 'common' phenotype which expresses GLUT-4 [11]. This is due to their increased 'recycling' of AfR or DHA with their increased intracellular ascorbate pool. After the loss of the GLO, all species that could absorb DHA into RBCs and regenerate electrons via the HMP to reduce extracellular DHA and quench ROS may have had an adaptive advantage. Individuals expressing GLUT-4 might not have been able to scavenge all radicals due to the absence of DHA transport, recycling and electron storage by intracellular vitamin C.

Interestingly, newborns of all species express GLUT-1 in their RBCs [10]. After three weeks, GLUT-1 could not be detected in the erythrocyte membrane of dogs, cows, rats and mice. This neonatal GLUT-1 expression enables the absorption of DHA and therefore the recycling of extracellular AA by RBCs in the first two to three weeks. In this period, the *de novo* synthesis of AA is not enabled [70].
The recommended daily allowances (RDA)s of species unable to synthesize vitamin C are significantly lower than the RDAs of species with artificial knock-out of AA biosynthesis: Nutrition of knock-out mice and rats must be supplemented with 200mg - 300mg AA per kg and day [4, 71] to prevent scurvy. In contrast, guinea pigs need about 2mg AA per kg and day [72] and humans only 1mg - 1.5mg vitamin C per kg and day [73, 4]. Newborn calves, which express GLUT-1 in their erythrocytes are in the same range need about 2-2.5 mg per kg and day[70]. The ODS rat has a maintenance dose of 10mg per kg [72].
The differences in the RDA of ODS rats compared to humans, guinea pigs and newborn calves might be due to different expressed GLUT transporters. ODS rats express GLUT-3, as previously stated, so, the higher RDA results from the competition between DHA and glucose for the transporter (fig. 3.4, page 25). The transport of DHA via GLUT-1 is completely glucose-concentration-independent, whereas the absorption by GLUT-3 competes with glucose. Vitamin C plasma levels increase after dietary intake of fruits, vegetables or root vegetables which also contain carbohydrates. Thus, both plasma vitamin C and plasma glucose concentrations rise after dietary intake. A competition of DHA with glucose results in decreased transport of DHA into RBC due to the concentration differences of plasma DHA ($<2\mu M$ [37, 32]) to plasma glucose (>8.8mM postprandial after consumption of raw or green leaf vegetables [74, 75]). Under normoglycemic conditions, the absorption of DHA was more than 3-fold lower in ODS rat RBCs than in human cells. Postprandial, this effect will be amplified further resulting in a 4-fold reduced intra-erythrocyte vitamin C concentrations (see fig. 5.2, page 58). A lack of competition between DHA and glucose for the transporter favors evolutionary success. Vitamin C intake occurs only in combination with dietary intake of carbohydrates, i.e. glucose. Therefore, the expression of GLUT-1 facilitated the absorption of DHA, whereas GLUT-3 did not and GLUT-4 transports no DHA at all.
The expressed transporter isoform is responsible for the intracellular concentration

vitamin C, and, therefore, the intra-erythrocyte electron pool. This ability for electron storage and the transfer of electrons cross over the membrane elucidates the differences in the different species' RDAs:
ODS rats have a 4-fold increased RDA of vitamin C compared to guinea pigs [72] (10mg/kg and day compared to about 2.5mg/kg and day). Via the HMP, up to 6-fold more electrons are provided compared to the AA biosynthesis (fig. 3.9, page 31). These regenerated electrons reduce the intracellular vitamin C molecules and can be disposed of when necessary. A 4-fold increased intra-erythrocyte ascorbate pool means that 4-fold more electrons can be stored and that in times with high oxidative stress 4-fold less vitamin is required in diet because of the oxidative defense dependent on the RBCs. Further, this electron transfer, and, therefore, the antioxidative defense also works in times when only little glucose is available.
To summarize, the dietary intake of vitamin C can decrease without any complications as long as the generation and storage of electrons within the RBC works.

The small differences in the RDA of newborn calves and guinea pigs compared to humans results from the loss of the urate oxidase (uricase) which metabolizes uric acid to allatonin in purine metabolism. Uric acid acts as a potent antioxidant in plasma by scavenging ROS [76] like vitamin C does. Due to the loss of urate oxidase, uric acid levels in plasma of humans and primates are increased [77]. It is possible that the loss of urate oxidase further decreases the RDA for vitamin C in humans due to the antioxidative nature of uric acid.

In conclusion, the expression of GLUT-1 and -3 in RBCs enables the absorption of DHA and increases the antioxidant defense of blood by recycling of extracellular oxidized forms of vitamin C. The recycling is energetically more efficient than the *de novo* synthesis of AA. Thus, also species with an active GLO might have a benefit, but no disadvantage by expressing one of these two transporter isoforms and could compete with other phenotypes that can explain the transport of DHA into rat RBCs.

3.2 Differences in the distribution of the membrane proteins GLUT-1 and stomatin in detergent resistant membranes of red blood cells from subjects with diabetes mellitus type 1 compared to healthy controls

In plasma AA is recycled by transfer of electrons across the erythrocyte membrane [68]. Frequently, decreased plasma vitamin C levels were observed in subjects with diabetes mellitus [46, 47, 48, 49]. Lower AA concentrations in plasma can be caused by either a higher production of DHA by oxidative stress and less absorption of this molecule, or from by a decreased recycling of AfR molecules by RBCs. In both hypothesis the transport of DHA into the RBC and its intracellular reduction plays a key role. The absorption of DHA can be affected by either a competition of DHA with glucose resulting in a smaller transport of DHA in normo- and hyperglycemic media, or by an altered interaction of GLUT-1 and stomatin which contributes to a decreased number of active DHA transporter proteins.

3.2.1 Effect of extracellular glucose on the distribution of GLUT-1 and stomatin in detergent resistant membranes

The effect of extracellular glucose on the GLUT-1 content of DRMs was examined (fig. 3.10, p. 35). In healthy subjects a significant negative correlation between GLUT-1 and the extracellular glucose concentration was observed (figure 3.11, page 36). Surprisingly RBCs from diabetic subjects show a significant lower GLUT-1 content in DRM than healthy cells do and no effect of extracellular glucose on GLUT-1 content can be detected.

These findings are supported by Meena Garg, who recently showed that RBCs of subjects with clinical type 1 diabetes contain less GLUT-1 molecules [78] in erythrocyte membrane. The differences in the absolute GLUT-1 number can explain the significant decreased amount of GLUT-1 molecules in DRM of diabetic subjects. A smaller total number of GLUT-1 proteins in RBC membrane results in a decreased

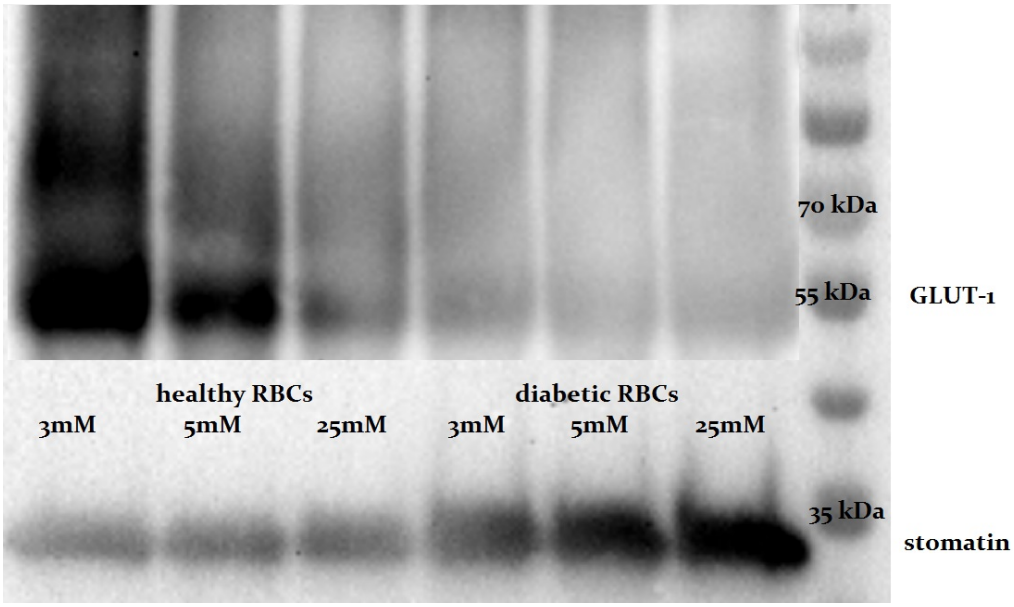

Figure 3.10: Representative Westernblot of GLUT-1 and stomatin in healthy (left 3 lanes) and diabetic (right 3 lanes) RBCs which were treated with different glucose concentrations (3mM, 5mM and 25mM respectively).

number of transporters which can be shuttled into DRM. But the smaller number of GLUT-1 molecules does not explain why diabetic cells do not respond to changes of the extracellular glucose concentrations. That could be first evidence for a missing regulation of GLUT-1 shuttle in diabetes.

In RBCs of healthy controls a transport of GLUT-1 molecules into DRM under hypoglycemic conditions was documented. These findings match the observations made by Anil Kumar, who reported a movement of GLUT-1 molecules into DRM after glucose deprivation[20]. The translocation of GLUT-1 resulted in an increased glucose transport activity due to the activation of inactive 'masked' transporters by shuttle into DRM, probably [21].
Consequently, the movement of GLUT-1 molecules represent a regulatory mechanism to ensure required energy uptake. This matches the observed findings (figure 3.11, page 36) of less GLUT-1 molecules in DRM under hyperglycemic conditions and more GLUT-1 in DRM in a hypoglycemic environment. A higher concentration of glucose molecules results in an increased chance for one molecule to find a transporter. When less glucose is available in plasma, the cell needs more active transporters to increase the likelihood for glucose uptake.

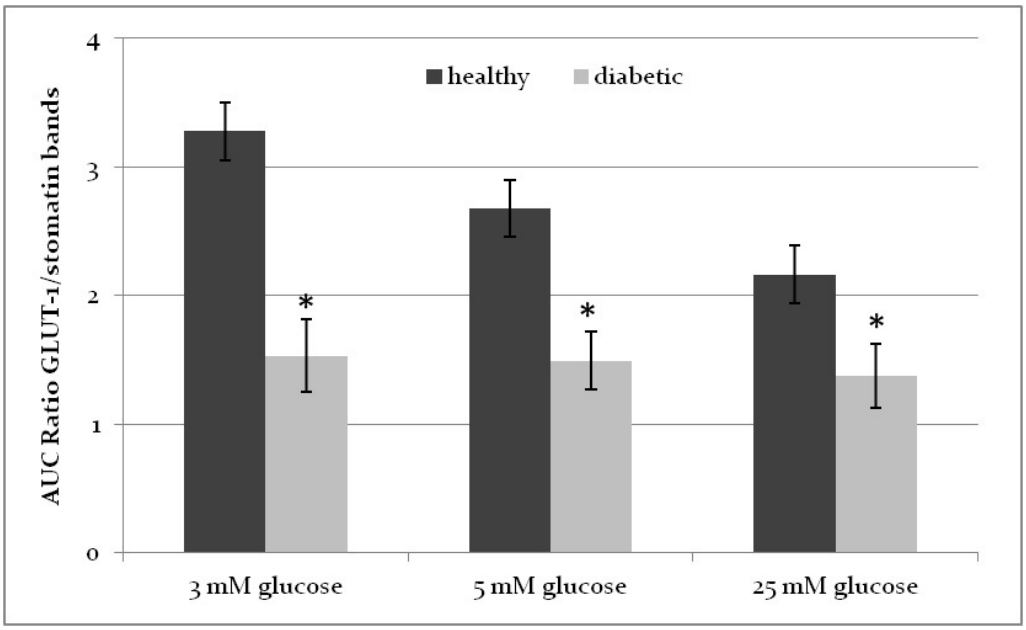

Figure 3.11: Ratio of GLUT-1 to stomatin bands of DRM isolated from RBC after incubation with 3, 5 or 25mM glucose (n=8). Significant differences ($p<0.05$) between the two groups are denoted by $*$.

In summary, the number of GLUT-1 molecules in DRM depends on the extracellular glucose concentration. But in diabetic samples no changes of GLUT-1 content was detected. Possibly, the regulation of glucose uptake into RBCs is disordered in diabetes. There are observations of an increase membrane microviscosity of diabetic RBCs which could possibly complicate GLUT-1 shuttling into DRM [79]. If RBCs from diabetic subjects can not shuttle their GLUT-1 into DRM, the uptake of DHA would be altered, too. If there is no shuttle of GLUT-1 into DRM, there is no possibility to interact with stomatin resulting in a decreased DHA uptake [11] and recycling [36]. Consequently, more DHA remains in plasma which is degraded and renaly excreted. Finally, the plasma ascorbate level decrease despite adequate dietary intake as frequently described [46, 47].

3.2.2 Effect of extracellular ascorbate on the distribution of GLUT-1 and stomatin in detergent resistant membranes

After treatment with glucose in combination with AA, the healthy samples showed small changes in DRM content of GLUT-1 compared to the samples treated with glucose only (figure 3.11, page 36). RBCs can not transport the reduced form of vitamin C [38] (see figure 3.1, page 22). So there is no need for the cells to shuttle more GLUT-1 into DRM than is needed for energy homeostasis.
Diabetic RBCs incubated with a combination of glucose and AA showed increased GLUT-1 movement into DRM compared to treatment with glucose only (glucose: $\sim$1.5 glucose and AA: $>$2.2).
Further, the GLUT-1 content significantly increased ($p<0.0002$) with the extracellular glucose concentration in DRM from diabetic RBCs. At a concentration of 25mM glucose, diabetic RBCs contained significantly more GLUT-1 in DRM than healthy controls did.

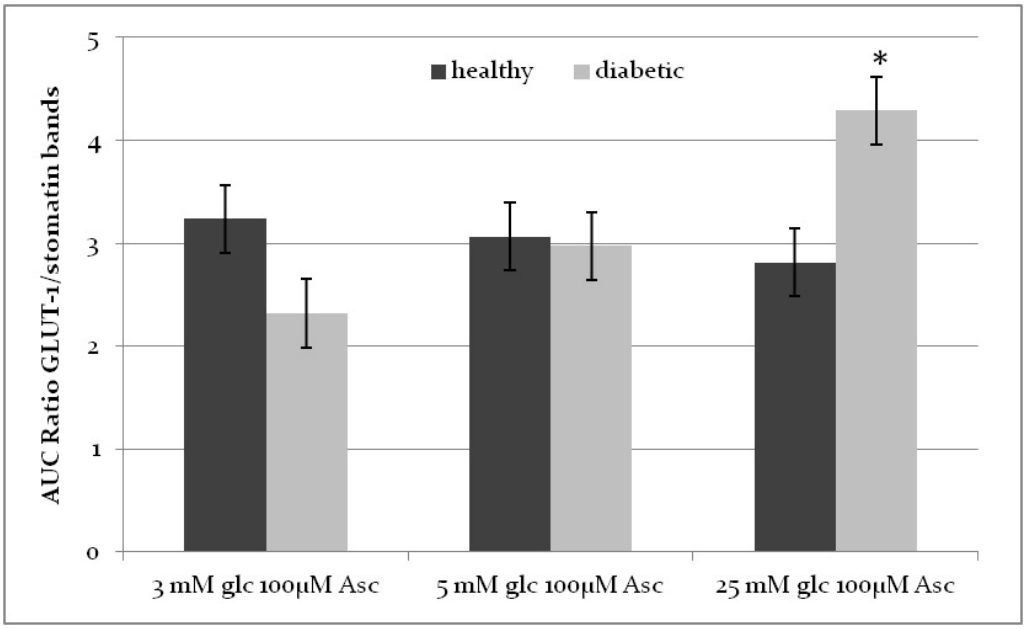

Figure 3.12: Ratio of GLUT-1 to stomatin bands of DRM isolated from RBC after incubation with 3, 5 or 25mM glucose and 100µM AA (n=6). Significant differences ($p<0.05$) between the two groups are denoted by $*$.

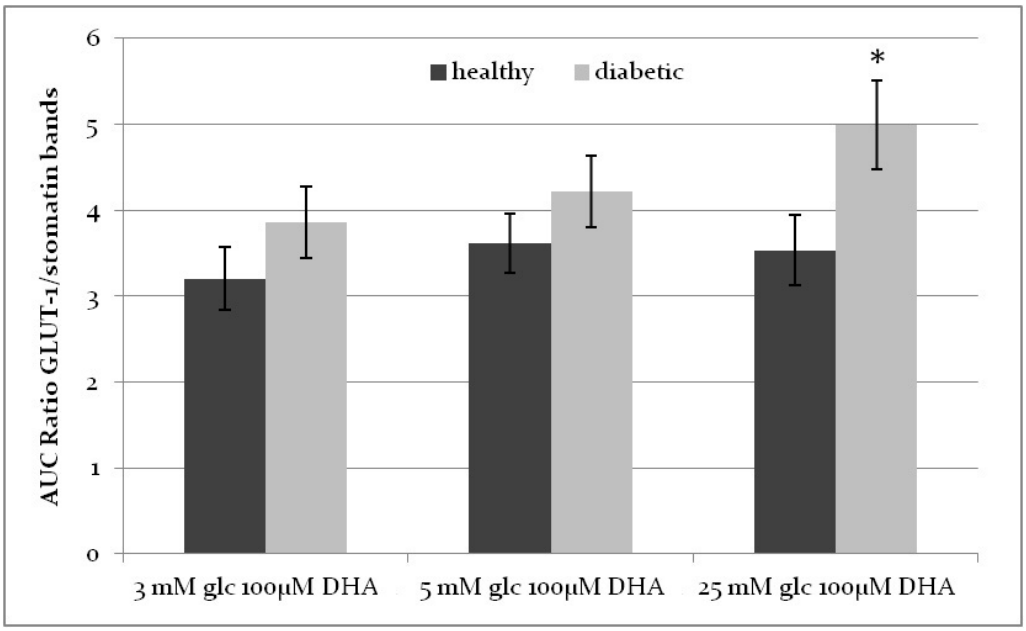

Figure 3.13: Ratio of GLUT-1 to stomatin bands of DRM isolated from RBCs after incubation with 3, 5 or 25mM glucose, 100µM AA and 5mU ascorbate oxidase to generate phsyiologic concentrations of DHA (n=6). Significant differences ($p<0.05$) between the two groups are denoted by $*$.

These results show that diabetic RBCs are able to move GLUT-1 into DRM. This shuttling is induced by extracellular ascorbate. This highlights the important role of this vitamin in diabetes mellitus type 1.

3.2.3 Effect of extracellular dehydroascorbate on the distribution of GLUT-1 and stomatin in detergent resistant membranes

In DRM from healthy controls which were treated with DHA and glucose no differences in GLUT-1 content were detected (figure 3.13, page 38). All treatments contained the same amount of DHA. A constant content of GLUT-1 demonstrates that healthy RBCs prefer the uptake of DHA over glucose as was suggested before by Montel-Hagen [11].

Diabetic RBCs showed the highest amount of GLUT-1 in DRM when incubated with 25mM glucose and DHA. In figure 3.12 on page 37, diabetic erythrocytes

contained the most GLUT-1 proteins when incubated in high glucose PBS and AA. This leads to the assumption, that diabetic RBCs are stimulated by the combination of high extracellular glucose concentration and vitamin C to translocate GLUT-1 into DRM. Hyperglycemic conditions favor the formation of ROS [80] resulting in a higher level of oxidative stress which is generally increased in individuals with diabetes mellitus [52, 53]. The movement of GLUT-1 might be an compensatory regulation to protect the vitamin from extracellular degradation.

For the recycling of the oxidized form of vitamin C, reduced glutathione (GSH) is required (see fig. 3.9 on page 31). For the recycling of an increased amount of DHA, the RBC also needs more glucose to generate the required reduction equivalents. There are observations of decreased net glucose transport via GLUT-1 transporter in patients with diabetes [81, 82]. Consequently, diabetic RBCs need more GLUT-1 proteins in DRM to transport the required glucose amount in addition to the number of GLUT-1 molecules needed for rapid DHA uptake. If the increased oxidative stress in diabetes favors the oxidation of AA to AfR and ultimately to DHA, the required number of GLUT-1 must be greater than in healthy cells.
Figure 3.14 on page 40 shows the content of GLUT-1 in DRMs from diabetic and healthy subjects sorted by extracellular glucose concentrations. It was observed that diabetic RBCs 'over-react' to changes in the extracellular AA and DHA concentration, especially under hyperglycemic conditions, compared to healthy controls.

In summary, the results from the human study show that diabetic RBCs are able to shuttle their GLUT-1 proteins into DRM. They regulate the GLUT-1 content in DRM dependent on the extracellular conditions, but only in combination with vitamin C. The decreased net glucose transport rate of GLUT-1 in diabetes [81, 82] is compensated for by increased transloction of GLUT-1 molecules into DRM. Especially under hyperglycemic conditions, GLUT-1 is shuttled into DRM. The result is likely improved DHA uptake and recycling of vitamin C. If this regulation works and the effect of extracellular glucose can be mediated by an increased GLUT-1 shuttling, the uptake rate of DHA into diabetic RBCs should be equal to that of healthy controls. Further, the transported amount of the oxidized vitamin should be equal at any glucose concentration as long as RBCs transport sufficient glucose to produce the required reduction equivalents needed for DHA recycling.

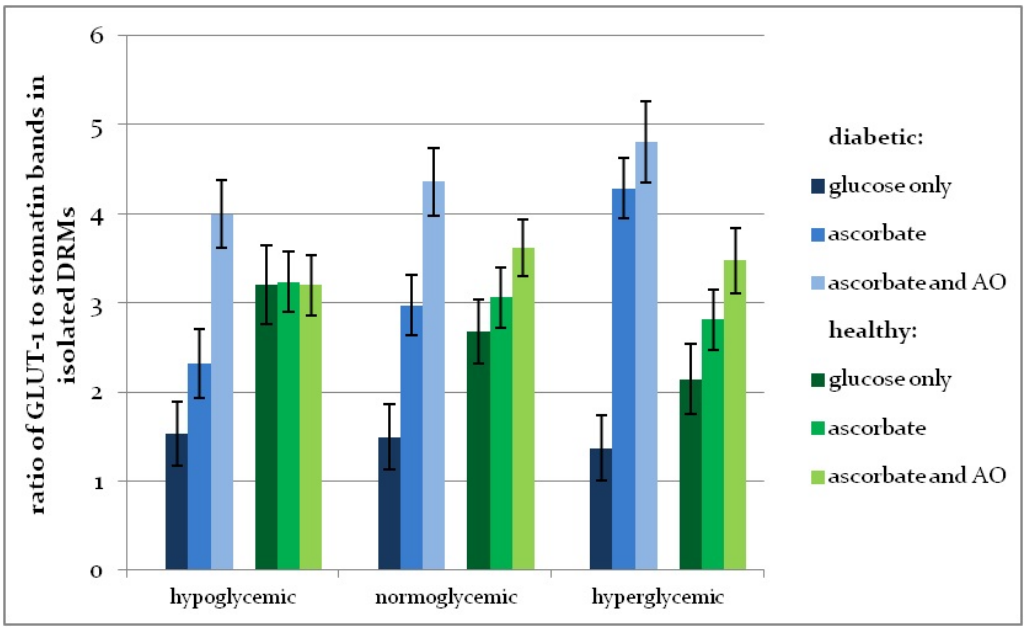

Figure 3.14: Ratio of GLUT-1 to stomatin bands of DRM isolated from RBCs of diabetic (blue bars) and healthy (green bars) subjects after incubation with 3, 5 or 25mM glucose and 100µM AA in combination with Ao to generate DHA. A statistical analysis for significances of the by glucose sorted data was not possible caused by the complex study design.

Uptake kinetics of DHA showed, that there are no differences in the transport of DHA into diabetic RBCs compared to healthy controls (fig. 3.15 on page 41). The extracellular glucose concentration had no effect on the transport of DHA. Thus, the altered GLUT-1 content found in diabetic DRM could compensate for the decreased net transport activity of GLUT-1 proteins and no competition of DHA with glucose was observed.

3.2.4 Ascorbate content of diabetic red blood cells *in vivo*

The intracellular AA concentrations of young and old RBCs from diabetic subjects were analyzed to examine whether the findings of equal DHA transport in RBCs *in vitro* can also be observed *in vivo*. Despite equal plasma vitamin C concentrations (fig. 5.1, page 57), diabetic RBCs show significantly less AA in young and middle-aged cells, the oldest RBCs of diabetic subjects also contained less intracellular

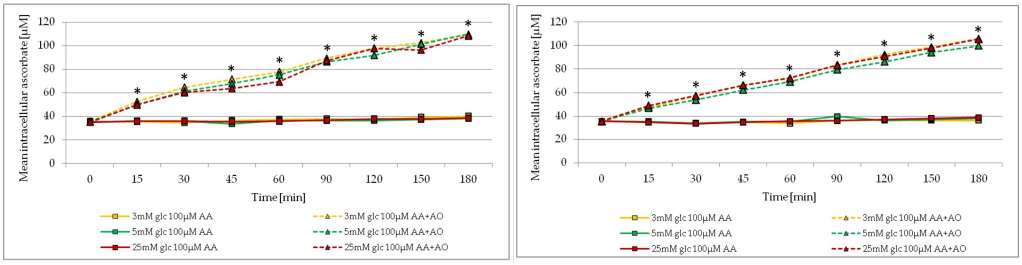

Figure 3.15: Accumulation of AA (continous lines) and DHA (broken lines) in RBCs obtained from healthy (left figure) and diabetic subjects (right figure). $*$ denotes significant differences in vitamin C treatment at the same glucose concentration and time. There are no statistical differences between the two groups.

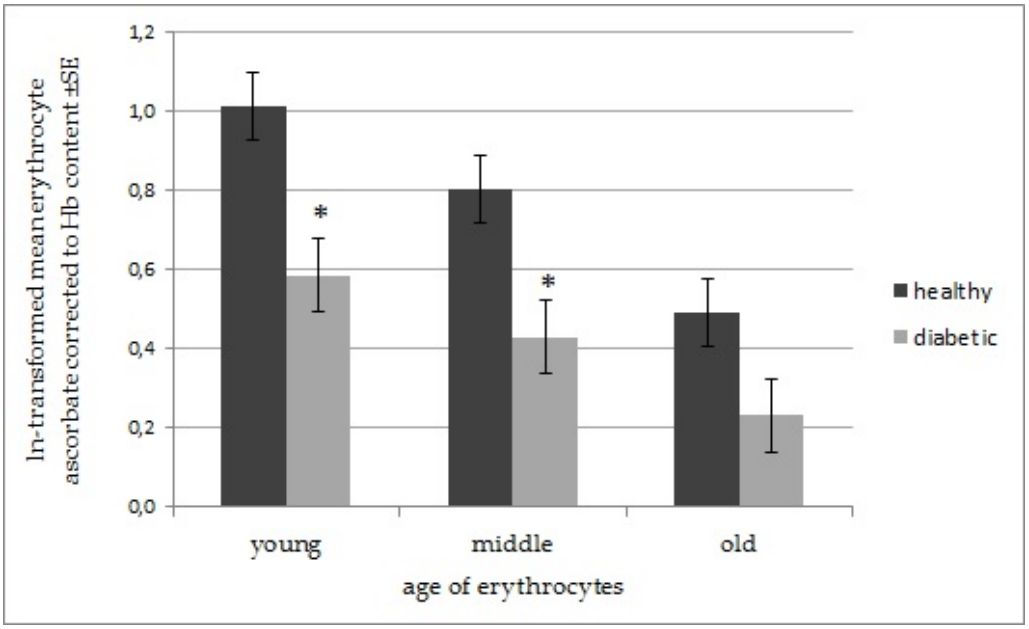

Figure 3.16: Intracellular AA concentration in healthy and diabetic RBCs separated by age (n=6). $*$ denotes significant differences for significances of the two groups (diabetics and controls).

ascorbate (figure 3.16, page 41). This demonstrates, that *in vivo* the transport of DHA into RBCs in diabetes mellitus is complicated. This leads to the assumption, that diabetic subjects have a smaller AA pool in their RBCs, and, therefore, a lower antioxidative defense compared to healthy controls. Thus, the compensation for the effect of extracellular glucose on DHA transport might be insufficient. The likely reduced DHA absorption *in vivo* results in decreased extracellular AA recycling by using intracellular electrons derived from ascorbate molecules. Thus, the reference daily intake (RDI) of vitamin C has to be reconsidered for diabetic patients to ensure an adequate antioxidative defense.

3.3 Limitations

In vitro research is always associated with problems regarding the provision of the right environment for cells and substrates. In the case of the uptake kinetic study, the main focus is on the stability of AA and a physiologic pH of the media. Thus, for all RBC uptake and incubation experiments phosphate buffered saline (PBS) has been chosen. Of course, plasma has many more components than PBS. Possibly, the metal ions and other micronutrients missing from PBS could influence the uptake of DHA *in vivo*.

DHA uptake kinetics performed in MCDB 131 cell culture medium showed a strong reduction in DHA uptake into RBCs compared to that performed in PBS (compare fig. 3.1, page 22 and fig. 5.3, page 59). Further, in *in vitro* uptake kinetics, no differences between healthy and diabetic subjects could be seen. But comparison of intracellular AA concentration of healthy and diabetic RBCs showed significant differences despite equal plasma ascorbate concentrations in both groups. The cells were analyzed immediately after blood withdrawal, thus artificial buffers or media could not affect the measured ascorbate concentration.

In summary, the results of this thesis provide a blueprint for how DHA transport into RBCs is modulated. While interpreting the data, it must be considered that biological systems are more complex and can not easily be replicated in the laboratory.

Chapter 4

Conclusion

Uptake kinetics of DHA into RBCs from man, pigs, Wistar and ODS rats showed that the classification into species with and without ascorbate synthesis gives no information about whether a species can transport the oxidized vitamin into its RBCs or not. Furthermore, it was documented that there are three different transport types, one which can not transport DHA, one whose DHA transport competes with the extracellular glucose, and one type in which the absorption of the vitamin is completely unaffected by the extracellular glucose concentration.
There are two known GLUT isoforms, GLUT-1 and GLUT-3, that are able to transport DHA. The altered absorption of DHA into RBC might result in the expression of different GLUT isoforms. Only GLUT-1 is known to translocate into DRM, and, therefore, to interact with stomatin which is the key to glucose-concentration-independent DHA transport. GLUT-3 can not interact with stomatin which accounts for the competition between DHA and glucose.
Fact is that all analyzed species which lost the ability to synthesize ascorbate express one of the DHA transporting GLUT isoforms. Consequently, the absorption of DHA into RBCs due to the expression of a different glucose transporter was likely the keystone event which led to the evolutionary survival of the GLO lacking species.

Further, it was demonstrated that RBCc are not a reservoir for vitamin C storage. The intra-erythrocyte vitamin C concentration decreases over the lifetime of these cells. Also, RBCs do not serve as a recycling compartment which absorbs the oxidized vitamin and disposes of the reduced form into plasma.
However, the absorption of DHA and the accumulation of ascorbate in RBCs

increases the antioxidative capacity in blood due to the transfer of electrons from intracellular ascorbate to extracellular AfR or DHA molecules. The benefit of using ascorbate as an electron donor might have been due to the fact that ascorbate is much smaller than NADPH+H^+ or GSH and therefore more molecules could be stored into an erythrocyte without loosing much intracellular space and without loosing much energy for the uptake.
In an energetic light, this recycling of plasma ascorbate is 4-6 fold more effective than the *de novo* synthesis of ascorbate under the assumption that vitamin C is available in diet. This calculations are supported by documentation of the RDAs of species, which lost the GLO. These species require less than 20% of the vitamin C artificial GLO knock-out species need.
The expression of GLUT-1 and GLUT-3 enables a species to recycle extracellular ascorbate by an electron transfer across the erythrocyte membrane. The evolutionary benefit of GLUT-1 expression was the interaction with stomatin resulting in a lack of competition between DHA and glucose for that transporter. Therefore, DHA is more effectively absorbed by RBCs and fewer molecules degrade and are excreted int eh urine. Due to the described extracellular ascorbate recycling, all species which express GLUT-1 in their RBCs have much smaller RDAs for vitamin C. This results in a better antioxidant defense which is more independent on the dietary vitamin C intake.

In diabetes mellitus type 1, lowered plasma vitamin C concentrations and decreased antioxidative capacities are frequently described. Analysis of GLUT-1 and stomatin content in DRM of erythrocytes showed an altered GLUT-1 regulation in subjects with diabetes mellitus. Significantly more GLUT-1 molecules are translocated into DRM under hyperglycemic conditions. Further, the intra-erythrocyte vitamin C concentration is significantly decreased.
The regulation of GLUT-1 localization into DRM is important for extracellular ascorbate recycling by RBCs. A decreased transport of DHA leads to a decreased number of intra-erythrocyte vitamin C molecules resulting in a decreased electron pool which can be transferred across the membrane. This leads to lower antioxidant defense in plasma and to an increased probability of oxidative damage. Further, a lower absorption rate for DHA results in an increased degradation and urinary loss of vitamin C.

To guarantee an adequate intra-erythrocyte electron pool, which is required for sufficient extracellular ascorbate recycling, the potential benefit of raising the RDI for vitamin C for diabetes patients should be investigated. A higher dietary intake possibly improves the absorption of DHA by RBCs and supports plasma ascorbate homeostasis.

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Chapter 5

Additional Results

5.1 Study population

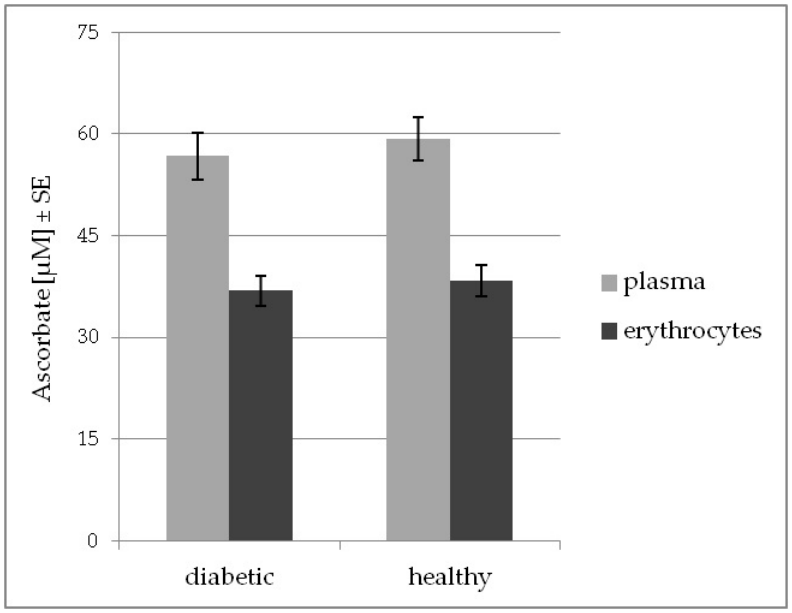

Figure 5.1: Mean plasma and erythrocyte ascorbate concentration of the study population. There were no differences in vitamin C concentrations between the two groups.

Table 5.1: Measured parameters of the diabetic subjects and healthy controls (± SE)

	diabetic	healthy
Age	32.5 ± 2.5	29.9 ± 2.5
HbA_1c	7.6 ± 0.2	5.2 ± 0.2
Total cholesterin	173 ± 7	189 ± 7
CRP	0.4 ± 0.5	0.9 ± 0.2

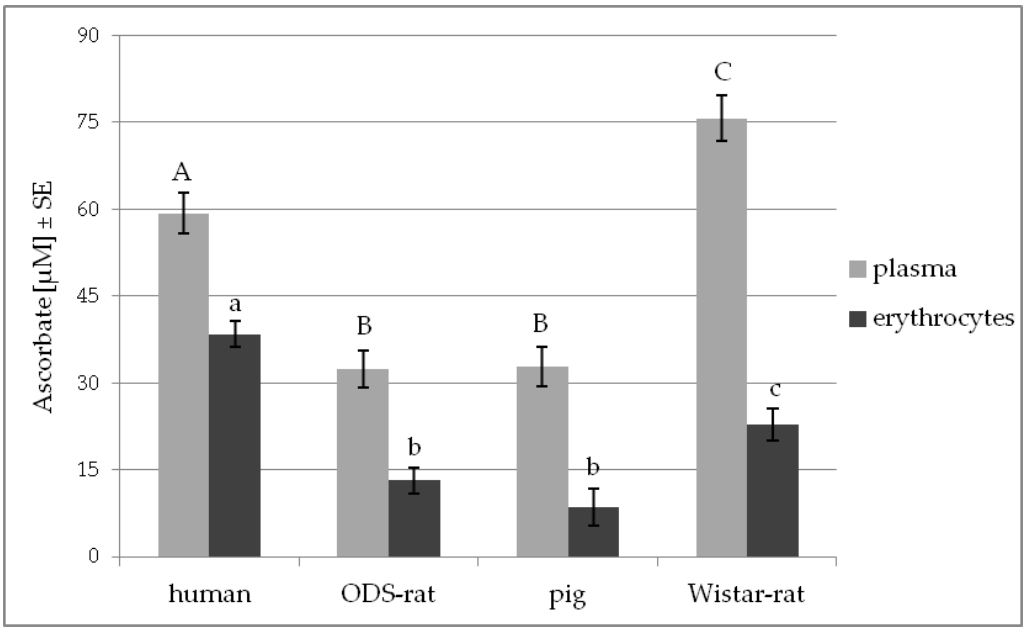

Figure 5.2: Mean ascorbate concentrations of plasma and erythrocytes obtained from different species. Bars with the same letter are not significantly different. Capital letters denote plasma values, small form letters denote cellular concentrations.

5.2 DHA uptake into HMEC-1 *in vitro*

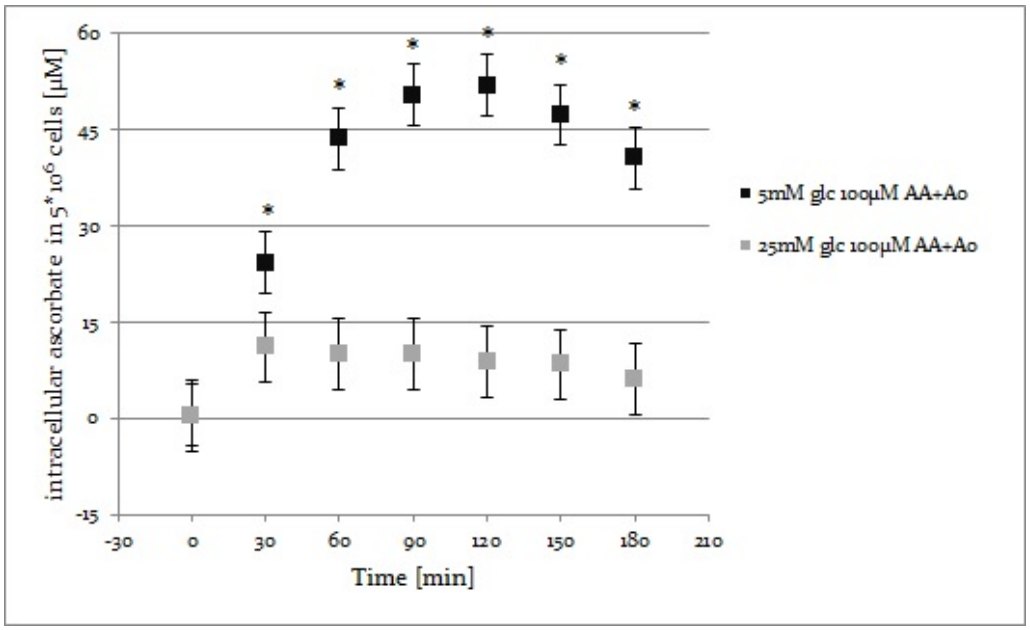

Figure 5.3: Uptake of DHA into HMEC-1 in media with 5mM or 25mM glucose. The transport and intracellular accumulation is significant less ($p<0.01$) in medium with high glucose concentration, significantly different datapoints are denoted by $*$. The measured AA concentration is generated by lysis of 5.5 x 10^6 in 300µl 5% PCA.

5.3 Westernblots of GLUT-1, GLUT-3 and stomatin

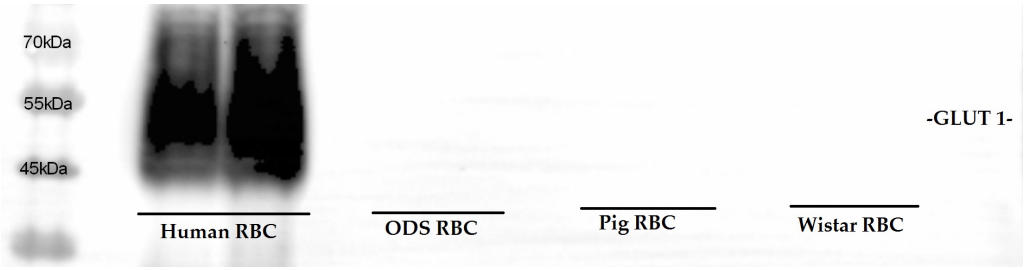

Figure 5.4: Representative Westernblot of RBCs obtained from men, ODS rats, pigs ans Wistar rats (from left to right). Samples from two individuals were analyzed, respectively. Only human RBCs contain GLUT-1, all other species were negative.

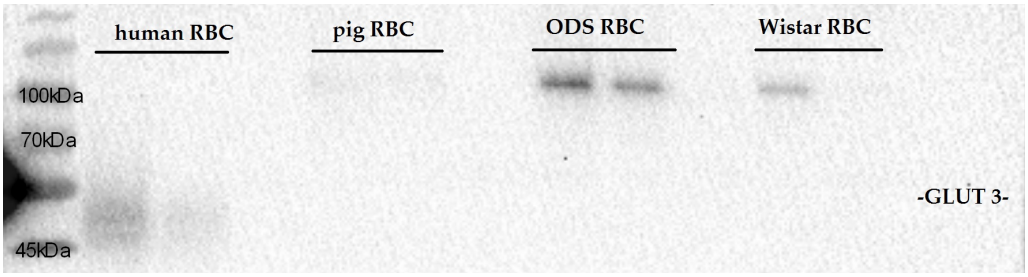

Figure 5.5: Representative Westernblot of RBCs obtained from men, pigs, ODS rats and Wistar rats (from left to right). Samples from two individuals were analyzed, respectively. No GLUT-3 was found in RBCs from human and pigs. Rat RBCs showed a band at 100kDa. The molecular mass of GLUT-3 is 50-55kDa [17]. The 100kDa band belongs to a co-localization of GLUT-3 with Hexokinase-1 like confirmed by Rauch *et al.* [83].

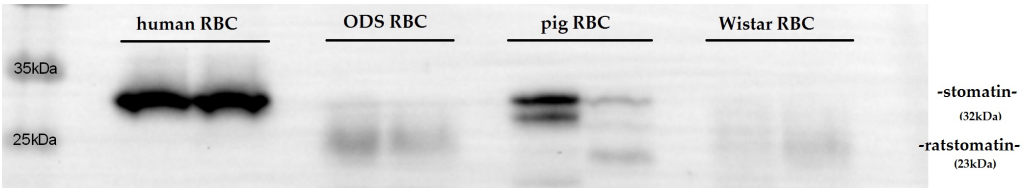

Figure 5.6: Representative Westernblot of RBCs obtained from men, ODS rats, pigs ans Wistar rats (from left to right). Samples of two individuals were analyzes, respectively. Rats express smaller stomatin molecules than men or pigs (cf. Wang *et al.* [84]). The double band observed in pigs belongs to a missing N-glycosylation which results in a 2-3 kDa smaller protein [85]

5.4 Graphics of transformed data with errorbars

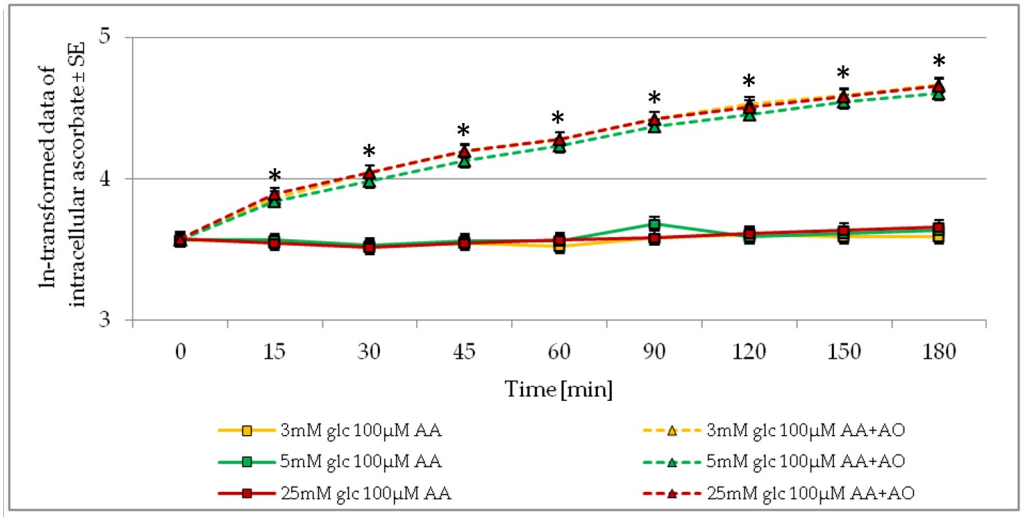

Figure 5.7: ln-transformed data of uptake kinetics into human RBCs

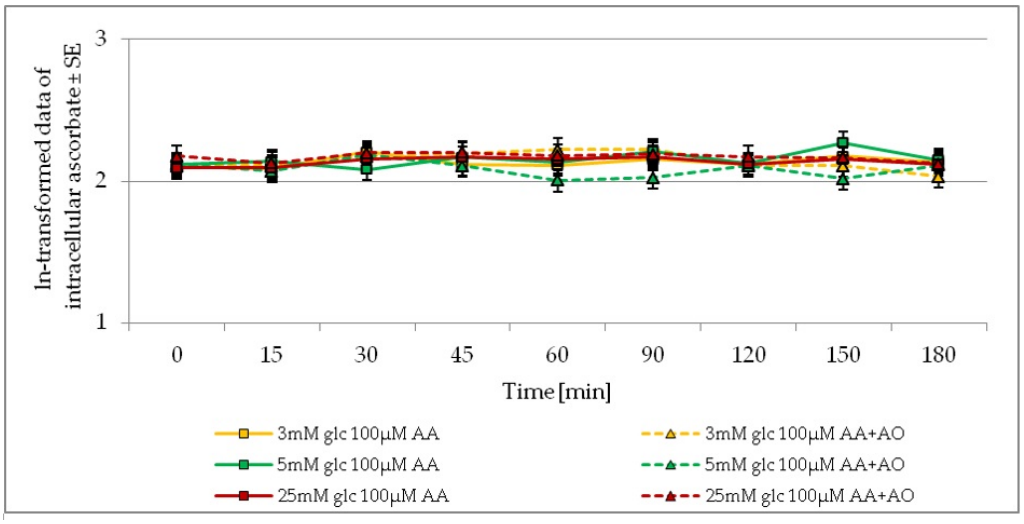

Figure 5.8: ln-transformed data of uptake kinetics into pig RBCs

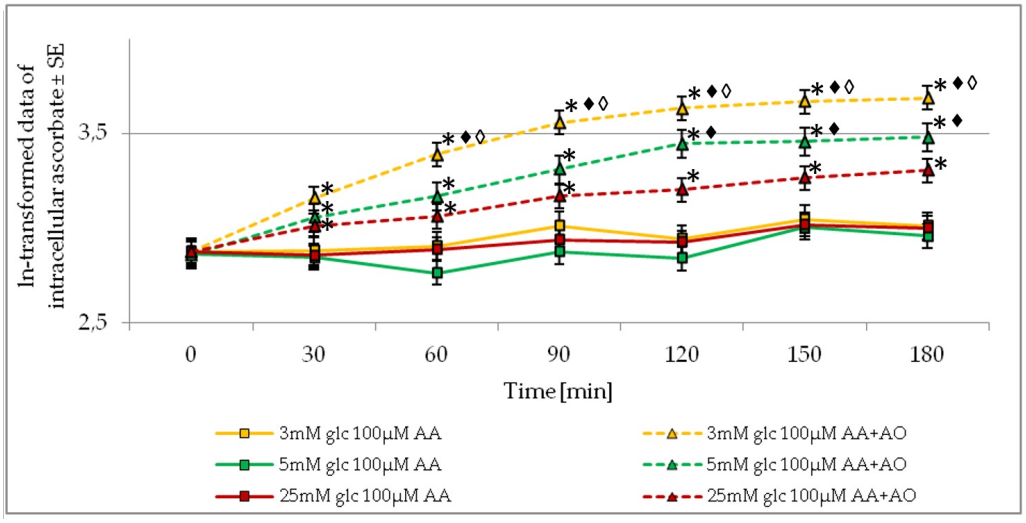

Figure 5.9: ln-transformed data of uptake kinetics into Wistar rat RBCs

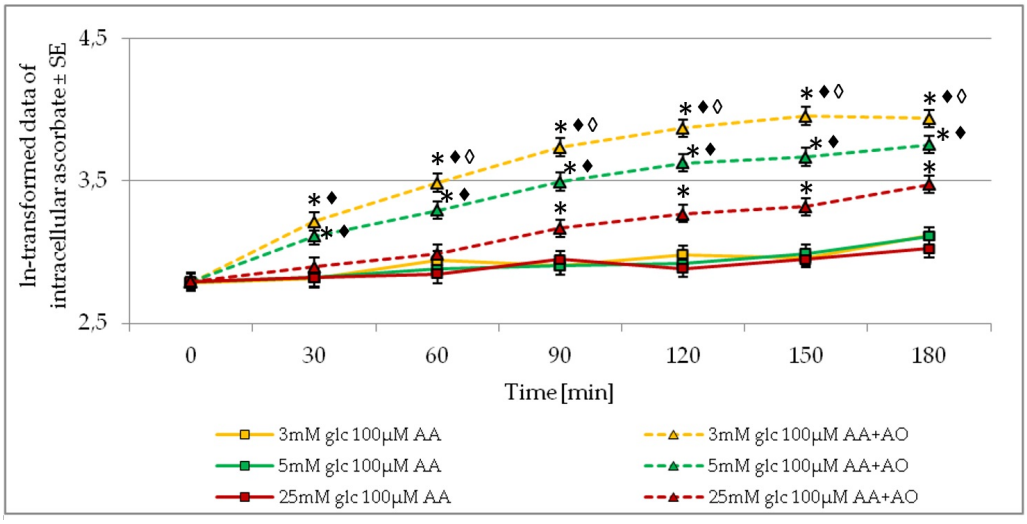

Figure 5.10: ln-transformed data of uptake kinetics into ODS rat RBCs

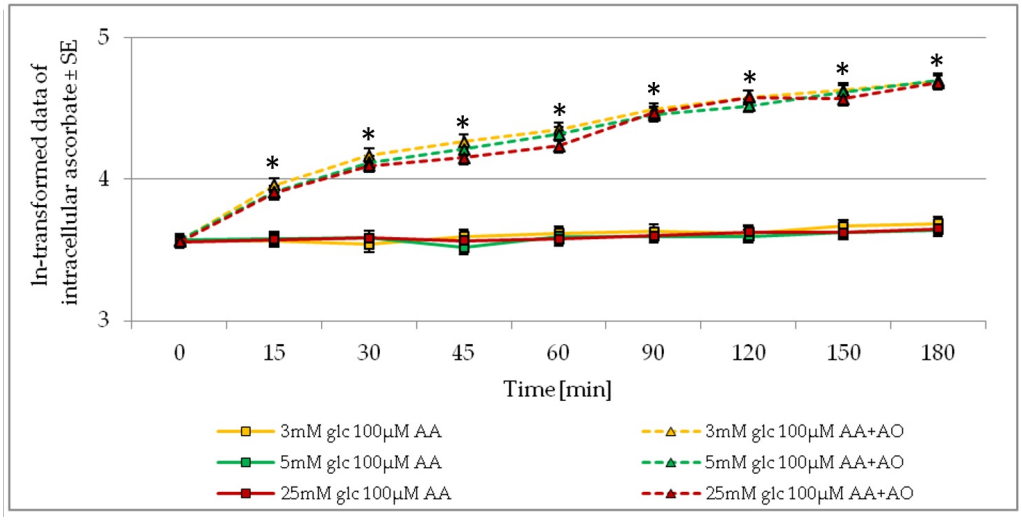

Figure 5.11: ln-transformed data of uptake kinetics into diabetic RBCs

Chapter 6

Materials and Compositions

Anode buffer A2

300mM/L Tris Base (CarlRoth AG, Karlsruhe, Germany) with 20% methanol (Carl-Roth AG, Karlsruhe, Germany).

Easycoll solution

Table 6.1: Manufacturing of Easycoll solution

density	volume Easycoll	volume PBS
1.081g/ml	6.39ml	3.91ml
1.092g/ml	7.82ml	2.18ml
1.099g/ml	7.90ml	2.10ml

Easycoll was purchased from biochrom GmbH, Berlin Germany.The solutions were mixed at room temperature, otherwise the transferred volumes were not correct.

Sørensen buffer

Sørensen buffer is a mixture of 0.5M Na_2HPO_4 (Merck KGaA, Darmstadt, Germany) solution with 0.5M NaH_2PO_4 (Merck KGaA, Darmstadt, Germany) solution. The ratio of the two solutions depends on the required pH. Table 6.2shows the volume of 0.5M Na_2HPO_4 solution which is mixed with 0.5M NaH_2PO_4 solution to a volume of 50ml resulting in a 0.5M sodium phosphate buffer with the required pH.

Table 6.2: Manufacturing of Sørensen buffer

pH	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
5									4.9	6.0
6	7.4	9.0	10.9	13.0	15.4	18.0	20.8	23.6	26.6	29.4
7	32.2	34.8	37.2	39.2	41.1	42.7	44.0	45.2	46.0	46.7
8	47.1									

Running buffer (Electrophorese buffer)

50mM/L Tris base (CarlRoth AG, Karlsruhe, Germany), 380mM Glycine (sigma-aldrich gmbH, Munich, Germany) and 0.1% sodium dodecylsulfate (serva electrophoresis GmbH, Heidelberg, Germany). The pH was adjusted to 8.5 - 8.7.

cathode buffer B

25mM Tris base (CarlRoth AG, Karlsruhe, Germany), 40mM aminocaproic acid (Merck KGaA, Darmstadt, Germany) and 20% methanol (CarlRoth AG, Karlsruhe, Germany)

Phosphate buffered saline (PBS)

137mM sodium chloride (Merck KGaA, Darmstadt, Germany), 2.7mM potassium chloride (Merck KGaA, Darmstadt, Germany), 1.5mM potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany) and 8mM disodium hydrogen phosphate dihydrate (Merck KGaA, Darmstadt, Germany). The pH was adjusted to 7.4 with hydrochloric acid.
For PBS-TX 0.5% Trinton-X100 (CarlRoth AG, Karlsruhe, Germany) was solved at room temperature in PBS.

PBS^- for cell culture

137mM sodium chloride (Merck KGaA, Darmstadt, Germany), 2.7mM potassium chloride (Merck KGaA, Darmstadt, Germany), 1.8mM potassium dihydrogen phosphate (Merck KGaA, Darmstadt, Germany) and 10mM disodium hydrogen phosphate dihydrate (Merck KGaA, Darmstadt, Germany). The pH was adjusted to 7.4 with hydrochloric acid.

Phosphatase and protease inhibitors

Phosphatase inhibitor stock solution was a 150mM sodium ortho vanandate solution. Aliquots were stored at -20°C until use.
Protease inhibitor Cocktail P8340 was purchased by sigma-aldrich (Munich, Germany). Aliquots were stored at -20°C like described by the manufactorer.

4xSDS sample buffer

8% sodium dodecylsulfate (serva electrophoresis GmbH, Heidelberg, Germany), 0.25M Tris base (solved and adjusted to pH6.8 before)(CarlRoth AG, Karlsruhe, Germany), 40% glycerol (CarlRoth AG, Karlsruhe, Germany), 0.003% bromphenole blue (sigma-aldrich gmbH, Munich, Germany), 20% β-mercaptho ethanol (Merck KGaA, Darmstadt, Germany). The volume was filled up with ultrapure Water to 10mL. Aliquots were stored at 4°C.

colorless 1xSDS buffer

2% sodium dodecylsulfate (serva electrophoresis GmbH, Heidelberg, Germany), 62.5mM Tris base (solved and adjusted to pH6.8 before)(CarlRoth AG, Karlsruhe, Germany) and 10% glycerol (CarlRoth AG, Karlsruhe, Germany). The volume was filled up with ultrapure Water to 40ml. Aliquots were stored at room temperature.

transporter substrates

L-Ascorbic acid was purchased by CarlRoth AG (Karlsruhe, Germany). To produce dehydroAA AA oxidase (sigma-aldrich gmbH, Munich, Germany) was added to an AA solution like described in the manufactorers instructions. Waterfree D-glucose was purchased from Merck (Darmstadt, Germany). Glucose stock solutions were stored up to 4 weeks at 4°C.

Tris bufferes saline with tween (TBST)

0.1M Tris base (CarlRoth AG, Karlsruhe, Germany), 0.37M sodium chloride and 0.05% Tween® 20 (serva electrophoresis GmbH, Heidelberg, Germany).
For blocking solution 5% bovine serum albumine (BSA)(CarlRoth AG, Karlsruhe, Germany) was solved in TBST.