Glucose transporter type 10—lacking in arterial tortuosity syndrome—facilitates dehydroascorbic acid transport

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Loss-of-function mutations in the gene encoding GLUT10 are responsible for arterial tortuosity syndrome (ATS), a rare connective tissue disorder. In this study GLUT10-mediated dehydroascorbic acid (DAA) transport was investigated, supposing its involvement in the pathomechanism. GLUT10 protein produced by *in vitro* translation and incorporated into liposomes efficiently transported DAA. Silencing of GLUT10 decreased DAA transport in immortalized human fibroblasts whose plasma membrane was selectively permeabilized. Similarly, the transport of DAA through endomembranes was markedly reduced in fibroblasts from ATS patients. Re-expression of GLUT10 in patients' fibroblasts restored DAA transport activity. The present results demonstrate that GLUT10 is a DAA transporter and DAA transport is diminished in the endomembranes of fibroblasts from ATS patients.

The Major Facilitator Superfamily consists several thousands of membrane transporters, including the *SLC2A* (solute carrier 2A) gene family encoding glucose transporter (GLUT) proteins. GLUT family members—presently 14 proteins—are grouped into three different classes based on their sequence similarities; GLUT10 encoded by *SLC2A10* gene belongs to class 3 [1]. Loss-of-function mutations in the *SLC2A10* gene are responsible for arterial tortuosity syndrome (ATS); however, the precise cellular location, the transported ligand(s), and the exact physiological role of GLUT10 have not been clarified.

Arterial tortuosity syndrome (OMIM #208050) is a monogenic autosomal recessive heritable connective tissue disorder characterized by elongation and generalized tortuosity of the major arteries. The clinical phenotype also includes aneurysms of large arteries and stenosis of the pulmonary artery. Patients usually present dysmorphic facial features and other connective tissue abnormalities such as hyperextensible skin and joint laxity [2–5]. Histopathological analysis shows extreme disorganization and fragmentation of elastic fibers in the arterial wall [2]. *SLC2A10* has been localized to human chromosome 20q12–q13.1, one of the genomic

Abbreviations
AA, ascorbic acid; ATS, arterial tortuosity syndrome; DAA, dehydroascorbic acid; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; NIDDM, noninsulin-dependent diabetes mellitus; ROS, reactive oxygen species.

loci associated with noninsulin-dependent diabetes mellitus (NIDDM), thus *SLC2A10* has previously been considered as a candidate gene for NIDDM [6]. However, independent studies have failed in confirming any association between *SLC2A10* mutations and NIDDM [7–9]. Three mechanisms have been proposed to explain the link between defective GLUT10 activity and the arterial defects described in ATS.

The first hypothesis, based on the perinuclear localization of GLUT10 in human fibroblasts, assumes that the glucose-dependent regulation of gene expression is altered in ATS. In particular, since in ATS cells the expression of decorin, a known proteoglycan TGFβ signaling inhibitor, was found down-regulated [2], it was supposed that the glucose-induced upregulation of decorin is missing in ATS, also explaining the upregulation of the TGFβ pathway observed in ATS cells [2].

The second theory suggests that GLUT10 is involved in the transport of dehydroascorbic acid (DAA), the oxidized form of the potent antioxidant ascorbic acid (AA) across the mitochondrial membranes, where GLUT10 has been localized with immunofluorescence studies in smooth muscle cells and adipocytes [10]. Upon being transported into mitochondrial matrix, DAA is reduced to AA, which can eliminate reactive oxygen species (ROS), thereby protecting the cell from oxidative stress. The ROS levels in the aortic smooth muscle cells of GLUT10 knockout mice [10] are indeed higher under oxidative stress conditions and their mitochondrial DAA transport activity is reduced. However, the relevance of these findings can be questioned as the phenotype of the GLUT10 knockout mice is very mild [11] or even normal [12] as compared to the human ATS phenotype.

A third hypothesis, based on our previous works [13–15] has also been proposed [16]. According to this hypothesis, GLUT10 acts as a DAA transporter in the endoplasmic reticulum (ER) membrane. DAA, in turn, is reduced to AA in the ER lumen, where it acts as a cofactor for the hydroxylation of prolyl and lysyl residues by Fe^{2+}/2-oxoglutarate-dependent dioxygenases, a biochemical reaction crucial for collagen and elastin maturation and folding. Supporting the hypothesis, it was shown that transiently transfected GLUT10-GFP constructs and organelle-specific fluorescent probe ER-Tracker Blue-White colocalized in rat aortic smooth muscle cells [16]. However, transport activities have not been demonstrated in the ER and other subcellular compartments.

As we demonstrated earlier, DAA is imported into the microsomal lumen by a transport system sharing the inhibitory properties of GLUTs [13] and DAA-derived AA accumulates in the ER lumen [14,15,17].

The transport activity was present not only in liver microsomes [13] but also in microsomes from various nonhepatic tissues/cells, including human fibroblasts [15].

Up to now, glucose or DAA transporters have not been molecularly characterized in the endomembranes. The presence of a glucose transporter has long been supposed as a component of the glucose-6-phosphatase system of the ER [18]. This assumption was experimentally confirmed using genetically encoded fluorescence resonance energy transfer nanosensors [19]. Glucose transport was shown to be mediated by at least two transporters with different characteristics in rat liver microsomes [20]. A possible explanation for this heterogeneity is that various plasma membrane GLUT transporters function locally during ER-to-plasma membrane transit [21]. Transport of DAA is typically mediated by GLUT transporters both in the plasma membrane and endomembranes [22]. Therefore, DAA transport in the ER can be also mediated by a GLUT transporter.

To date, there is no consensus regarding the subcellular localization of GLUT10 and the ligand it transports. On the other hand, GLUT10 could excellently act the part of the missing glucose/DAA transporter of the ER. Therefore, the aim of this study was to verify the role of GLUT10 as a DAA transporter. Our observations demonstrate that GLUT10 is functioning as a DAA transporter and DAA transport activity is diminished in the endomembranes of fibroblasts from ATS patients lacking functional GLUT10 transporter.

Materials and methods

Patients and cell cultures

Skin fibroblasts from ATS patients and unrelated healthy donors were established from skin biopsies as previously reported [2]. Patient 1 (P1) was homozygous for the c.1334delG microdeletion [2]; patient 2 (P2) was compound heterozygous for the c.1309G>A and the c.1330C>T transitions [23]; patient 3 (P3) was homozygous for the c.1411+1G>A splicing mutation [24]; and patients 4–8 (P4–P8), members of the same family, all bearing the homozygous c.510G>A mutation [2]. Written informed consent was obtained from each patient for skin biopsy procedure. This study was approved by the medical ethical committee of the University Hospital Spedali Civili of Brescia, Italy (in case of P1–P3) and by the Ghent University Hospital, Belgium (for P4–P8), and was performed in accordance with the Declaration of Helsinki Principles.

Dermal fibroblasts cultures were grown *in vitro* at 37 °C in a 5% CO_2 atmosphere in Earle's Modified Eagle Medium (MEM) supplemented with 2 mM L-glutamine, 10%

FBS, 100 μg/mL penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were analyzed at the same *in vitro* passage number (5th to 7th).

Construction of a stable GLUT10 expression vector

A stable GLUT10 expression vector was generated as previously reported [25]. Briefly, the full length of human *SLC2A10* cds (refseq: NM_030777.3) was amplified from total RNA of normal skin fibroblasts with the SuperScript III One-Step RT-PCR System and inserted into the eukaryotic pEF6/V5-His-TOPO™ expression vector containing the blasticidin-selectable marker gene and the strong human elongation factor 1α promoter (Life Technologies). This expression vector (pG10) and the empty cloning vector (mock) were stably transfected in the skin fibroblasts of P1 using the FUGENE 6 liposomal transfection reagent (Roche Applied Science, Mannheim, Germany). Selection was achieved by adding selective medium (2 μg/mL of blasticidine) that was changed every 3 days until the elimination of all sensitive cells. Established cell lines carrying an integrated pG10 construct or empty vector were expanded for subsequent experiments.

Indirect immunofluorescence analysis

To analyze the presence and distribution of GLUT10, control, untransfected and mock-transfected ATS fibroblasts, and ATS cells re-expressing GLUT10 were grown for 48 h and reacted for 2 min with 3% paraformaldehyde/0.5% Triton, 20 min with 3% paraformaldehyde, washed with 100 mM glycine/PBS, blocked for 30 min with 5% BSA, and immunoreacted overnight at 4 °C with 20 μg/mL polyclonal rabbit anti-GLUT10 antibody (Alpha Diagnostic Int. Inc., San Antonio, TX, USA). After washing in PBS, cells were incubated for 1 h at room temperature with the rhodamine-conjugated anti-rabbit secondary antibody. The signals were acquired by a CCD black and white TV camera (SensiCam-PCO Computer Optics GmbH, Kelheim, Germany) mounted on a Zeiss fluorescence Axiovert microscope, and digitalized by Image Pro Plus program (Media Cybernetics, Silver Spring, MD, USA). The experiments were repeated three times.

Subcellular fractionation of human fibroblasts

Microsomal fraction was prepared from human fibroblasts as reported earlier [26] with minor modifications. The trypsinized cells were resuspended in sucrose-HEPES buffer (0.34 M sucrose, 10 mM HEPES; pH 7.4) and sonicated five times for 15 s at 4 °C using Sonic 300 Dismembrator (35% pulse cycle). Cell homogenates were centrifuged for 10 min at 1000 *g*. The postnuclear supernatant were centrifuged

for 20 min at 18 000 *g*. Microsomes were recovered by ultracentrifugation for 60 min at 195 000 *g*. Pellets were resuspended in 20 mM MOPS buffer (pH 7.2) and maintained in liquid N_2.

hTERT fibroblast cultures

BJ-5ta hTERT immortalized fibroblasts from LGC Standards (Teddington, Middlesex, UK) were cultured in a 4 : 1 mixture of Dulbecco's Modified Eagle Medium (Life Technologies) and Medium199 (Life Technologies) supplemented with 0.01 mg/mL hygromycin B (Life Technologies), 10% FBS (Life Technologies) and 1% Antibiotic–Antimycotic (Life Technologies). All cultures were grown in 75-cm² cell culture flasks (Sigma-Aldrich Co., Saint Louis, MO, USA) and maintained in a humidified incubator at 37 °C with a 5% CO_2 in air atmosphere.

Silencing

shRNA-mediated knockdown of the *SLC2A10* gene was achieved with human GIPZ lentiviral shRNAmir constructs containing viral particles (Costume-made by Thermo Fisher Scientific Rockford, IL, USA). Three different silencing sequences and a scrambled sequence were cloned in lentiviral vectors to produce viral particles. Infection of hTERT cells was performed according to the manufacturers' instructions (Thermo Fisher Scientific—Technical Manual —Expression Arrest GIPZ Lentiviral shRNAmir), with a multiplicity of infection (MOI) of 3 : 1. For stable expression experiments, selection of transduced cells was performed with puromycin, starting 48 h after infection.

RNA isolation and PCR analysis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Two micrograms of RNA were reverse transcribed with SuperScript® III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific) and random hexamers. Expression levels of *SLC2A10* were quantified by fluorescent real-time PCR with a DNA engine thermal cycler (MJ Research, Waltham, MA, USA) equipped with Opticon Monitor 4 software. Analyses were performed in triplicates in a 25-μL reaction mixture. cDNA (1 μL) was amplified with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 200 nM of the sense and antisense primers. For *SLC2A10*, the oligonucleotide primers were: sense, 5′ CTTCAACTGGGCGGCCAACCT3′; antisense, 5′ CGAGGACAGCGGTCAGTCCGT3′. Amplification protocol was: 95 °C (10 min), 40 cycles of 95 °C (20 s), 55 °C (20 s), 72 °C (20 s). The PCR amplification efficiency was evaluated by serial (10- fold) dilutions of the human cellular cDNA. Diluted and undiluted samples were

then analyzed in duplicate. Amplification efficiency was calculated as reported [27]. The amplification efficiency was more than 90%. Every assay was run in triplicate and negative controls (no template, template produced with no reverse transcriptase enzyme) were always included. In the negative controls, no signal was detected in the investigated amplification range (40 cycles).

Western blot analysis

For western blot analysis, hTERT cells were subcultured in a 6-well plate, and used after 2–3 days. Confluent wells were washed with PBS and lysed with RIPA buffer (50 mM Tris-Cl, pH8, 150 mM NaCl, 1% NP40, 0.1% SDS). Total protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal protein amounts (15–20 μg) were subjected to 10–12% SDS/PAGE and transferred to polyvinylidene difluoride membranes by semidry western blotting. The membranes were blocked in 5% milk or 5% BSA overnight, then probed using specific rabbit polyclonal anti-glucose transporter GLUT10 antibody (1 : 1000; Abcam, Cambridge, UK) and GAPDH (1 : 7500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The western blots were developed using chemiluminescent detection system (Thermo Fisher Scientific). Protein levels were evaluated through densitometry.

Preparation of cells for transport measurements

Cells grown in 75-cm^2 cell culture flasks, were detached by trypsinization, harvested by centrifugation (5 min; 600 *g*), washed, recentrifuged, and resuspended in sterile PBS (2×10^6 cells/mL).

Plasma membrane-permeabilized fibroblasts were prepared as described above, except that following the last centrifugation step cells were resuspended in MOPS buffer (100 mM KCl, 20 mM NaCl, 1 mM MgCl$_2$, 20 mM MOPS, pH 7.2). Having counted the cells, suspensions were incubated at 4 °C in the presence of 1 mM NaN$_3$ and 40 μM digitonin until permeabilized (10–20 mins). Plasma membrane permeabilization was controlled by trypan blue exclusion. After removal of digitonin by centrifugation, cells were used for transport assays.

Liposome preparation

Large unilamellar liposomes (LUV) were prepared from L-α-phosphatidylcholine from egg yolk (egg-lecithin, Cat. No. 61755; Sigma-Aldrich Co.). Appropriate amount of lipids, dissolved in chloroform-methanol (9 : 1) mixture, was dried as a thin film on the wall of a glass tube under argon stream and the traces of the organic solvents were further removed in a vacuum desiccator at 2 Hg mm for 30 min. Multilamellar liposomes were prepared at 40 °C in 20 mM

Hepes buffer (pH 7.5) supplemented with 10 mM GSH in a lipid concentration of 20 mg/mL. LUV were obtained by extrusion of the multilamellar vesicle suspension through a polycarbonate filter (pore size 0.4 μm, Nuclepore Track-Etch Membrane; Whatman International Ltd.; Maidstone, Kent, UK). At least 40 extrusion cycles were performed at 40 °C (the extruder and the filters were purchased from Avanti Polar Lipids Inc. Alabaster, Alabama USA). Proteoliposomes were prepared from and transport experiments were performed on freshly prepared LUVs. For the preparation of proteoliposomes, 5 μL of liposomes (20 mg L-α-phosphatidylcholine/mL) was fused with 10 μg of *in vitro*-translated GLUT10 protein or 60 μg of microsomal protein.

In vitro translation of GLUT10 transporter

Human *SLC2A10* gene was amplified from GLUT10 vector (pLenti-suCMV-hSLC2A10, Rsv RFP-Puro, 150 ng/μL, Amsbio) by PCR using primers 5′TACTTCCAATCCAAT GCAATGGGCCACTCCCCACCT3′ and 5′TTATCCACT TCCAATGCATCAGGAGGCCGCAGAGAT3′. The PCR product was transferred into pEU3-NII-GLICNot vector by ligation-independent cloning. The vector construct is a modification of wheat germ translation vector [28] and codes the recombinant protein with TEV protease cleavable GST affinity tag. *Escherichia coli*-competent cells were transformed with these GLUT10 possessing vectors. Plasmid purified from ampicillin-resistant colony was sequenced to confirm the PCR accuracy.

The *in vitro* mRNA synthesis was accomplished by the addition of 1 μg of purified, NotI-linearized vector following the provided manual of the kit (TranscriptAid T7 High Yield Transcription Kit; Thermo Fisher Scientific). The reaction was incubated for 2 h at 37 °C and then precipitated by ammonium acetate/ethanol mixture. The mRNA pellet was washed with absolute ethanol, air-dried, and dissolved in SUB-AMIX buffer of cell-free translation.

The batch-wise translation mixture contained 5 μL wheat germ extract (Cell Free Sciences), 1 μg mRNA, 5 μL liposome (20 mg/mL), 0.8 μL creatine kinase (1 mg/mL), and was completed to 25 μL final volume with 1× SUB-AMIX solution [29]. The reaction was incubated at room temperature for 3 h. The control reactions were set up either without liposome or exogenous mRNA. The reaction mixtures were centrifuged at 10 000 *g* for 30 min and both the supernatant and the pellet were analyzed by western blot. It was observed that the presence of liposomes resulted in more effective translation and less aggregation of GLUT10 protein.

Transport assays

The uptake/transport of DAA, AA, and other compounds was investigated by a rapid filtration method with trace

amount of radiolabeled compounds as described previously [13]. Intact and plasma membrane-permeabilized cells incubated in the presence of the indicated ligands and their radiolabeled analogs for the indicated times were filtered through glass fiber paper GF/C; 2.5 cm; pore size 0.45 μm (Whatman International Ltd.). Filters were washed with 2 mL of the medium and the radioactivity retained on the filter was measured by liquid scintillation counting. In each experiment, deoxycholate (0.4% final concentration) was added at the end of the incubations; the radioactivity not released by this maneuver was regarded as binding and was subtracted from the measured values.

Transport into microsomes and proteoliposomes was measured by the same method as described in details earlier [13]. Since the yield of microsomal fraction was poor from fibroblasts, microsomal vesicles were fused with liposomes and transport assays were performed on the resulting chimeric vesicles.

The following radiolabeled compounds were used in the experiments: D-[U-^{14}C]Glucose; (Cat. No.: CFB2); Amersham Biosciences UK Ltd.; Little Chalfont, Buckinghamshire, UK; [U-^{14}C]Sucrose; (Cat. No.: CFB146); Amersham Biosciences UK Ltd.; Uridine Diphosphate Glucuronic Acid, [Glucuronyl-U-^{14}C]; (Product No.: NEC414010UC) Perkin Elmer; Boston, MA, USA; Ascorbic Acid, L-[1-^{14}C]; (Cat. No.: ARC1569); American Radiolabeled Chemicals, Inc.; 101 ARC Drive, Saint Louis,

MO, USA. DAA was prepared by the bromine oxidation method as reporter earlier [13].

Measurement of AA/DAA by HPLC

Intra- and extracellular total vitamin C levels (AA + DAA) were determined by HPLC [30]. Cells were incubated in the presence of 50 μM AA for 0, 6, 12, 24 h. After incubation, the cells were washed twice with PBS and scraped with rubber policeman in 25 w/v% metaphosphoric acid solution. To measure AA and DAA together, 5 mM DTT (final concentration) was added to the samples and they were incubated for 20 min at room temperature. Before HPLC measurement, samples were centrifuged at 3000 *g* for 10 min. Vitamin C was measured from the clear supernatant. Samples were eluated through the column (Teknokroma NUCLEOSIL 100 C18 5 μm, 25 × 0.46 mm) with 0.1 M Sodium-Dihydrogene-Phosphate solution (pH 3.1) containing 0.2 mM EDTA. Vitamin C content of the medium was determined using the same method.

Results

GLUT10 is a DAA transporter

In the first set of experiments, DAA transport was measured in GLUT10-containing proteoliposomes. GLUT10 protein was produced in an *in vitro*
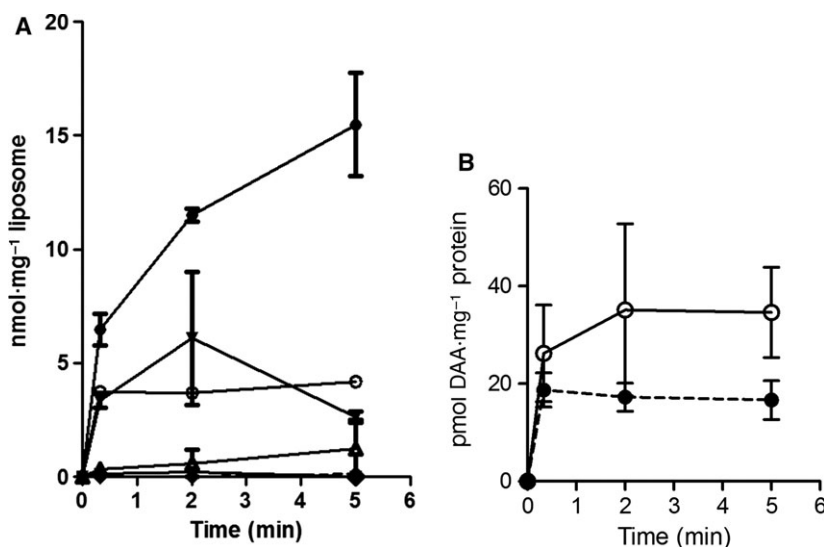

Fig. 1. GLUT10 protein acts as a DAA/glucose transporter in proteoliposomes. (A) GLUT10 protein was produced by an *in vitro* translation method and was incorporated into liposomes. Transport activities were measured by a rapid filtration method and radiolabelled ligands. The following ligands were used (in 1 mM concentration if not indicated otherwise): DAA (O); DAA 5 mM (●); AA (△); glucose (▼); UDP-glucuronic acid (⊗); sucrose (□). Transport of protein-free liposomes with 1 mM DAA was also measured (◇). (B) DAA transport (1 mM) was measured in proteoliposomes gained by the fusion of microsomes prepared from the fibroblasts of healthy controls (O) and ATS patients P4 and P7 (●) with liposomes. Results are expressed as means ± SEM of four experiments or (where SEM bars are missing) as means of two measurements.

Fig. 2. Silencing of GLUT10 in hTERT-immortalized human fibroblasts decreases DAA transport through the endomembranes. Silencing of GLUT10 in human hTERT fibroblasts were executed with lentiviral-based silencing vectors expressing three different shRNA sequences. Out of the three constructs, two (shRNAII and shRNAIII) were proven to be effective in silencing the transporter with RT-PCR analyses (A). Effective silencing was also verified by immunoblotting at protein level (B). Right panel shows one representative western blot out of four; the result of densitometric analysis is presented on the left panel. GAPDH is used as housekeeping marker. Examination of DAA uptake (1 mM) in mock silenced and effectively silenced cell lines was performed with rapid filtration technique (C). DAA transport significantly decreased in both cell lines that were effectively silenced. Results are expressed as means ± SEM of 5–10 experiments. Correlation between GLUT10 protein levels and DAA transport activities in mock silenced and effectively silenced cell lines is shown in (D). Data are taken from panels B and C. Asterisks mark statistically significant differences from mock transfected (*$P < 0.05$; **$P < 0.01$).
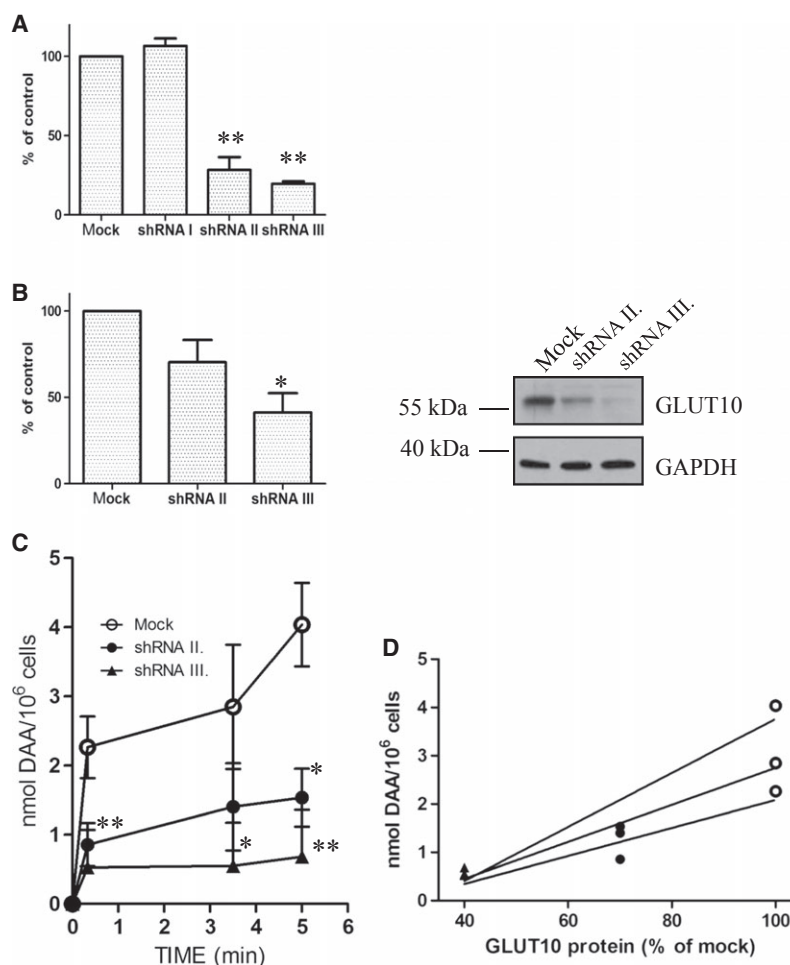
translational system and the protein was incorporated into liposomes to exclude the eventual indirect effects of the lack of GLUT10 on DAA transport. The presence of GLUT10 in the proteoliposomes was checked by immunoblotting (data not shown). GLUT10-containing proteoliposomes efficiently transported DAA in a concentration-dependent way (Fig. 1A). Glucose transport was also measurable, while unrelated compounds such as UDP-glucuronic acid and sucrose were not transported. A very low AA uptake was also observed, which was presumably due to the oxidation of AA to DAA during the incubation [14]. Incorporation of microsomal proteins prepared from human control fibroblasts into liposomes also promoted the transport of DAA (Fig. 1B). Protein-free liposomes did not show transport activities (Fig. 1A).

Silencing of GLUT10 compromises DAA transport

To demonstrate the involvement of GLUT10 in subcellular DAA transport, a hTERT immortalized human fibroblast cell line was silenced for GLUT10 with lentiviral-based silencing vectors. Stable clones expressing the silencing vector were selected and were checked for the efficiency of silencing. Semiquantitative RT-PCR analysis of the stable clones showed that two silencing constructs out of three were effective (Fig. 2A).

Immunoblotting of cell homogenates revealed that only one of the constructs decreased GLUT10 at protein level effectively (Fig. 2B). Measurement of DAA transport on plasma membrane-permeabilized hTERT immortalized human fibroblasts showed that it was severely decreased in these efficiently silenced cells (Fig. 2C). Strict correlation (R^2 values between 0.8859 and 0.9967) was observed when DAA transport activities were plotted against GLUT10 protein levels detected by western blot in silenced fibroblasts (Fig. 2D).

DAA transport through endomembranes is defective in ATS

The uptake of DAA and its reduced form, AA, was investigated in intact human fibroblasts from healthy
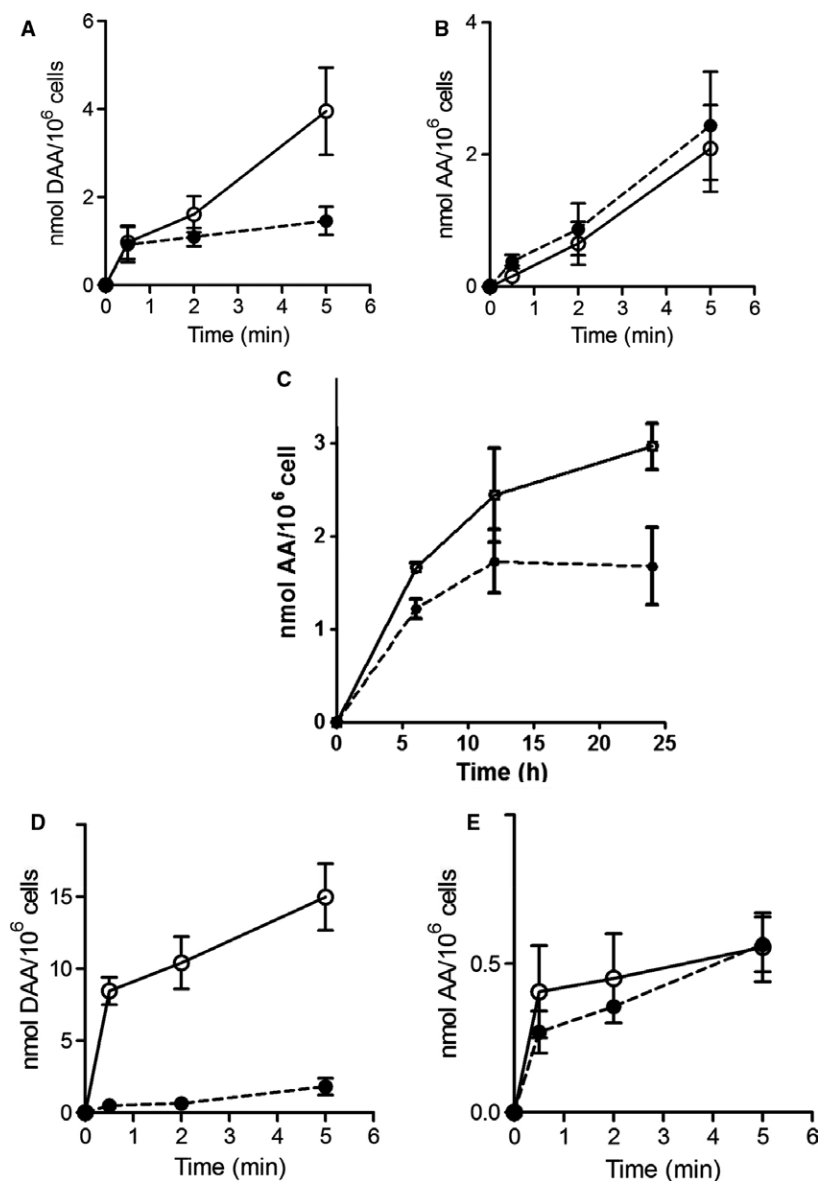
Fig. 3. DAA transport through endomembranes is defective in ATS. DAA (A) and AA (B) uptake was measured in intact control (empty circles) and ATS fibroblasts (black circles) using a rapid filtration method and radiolabeled ligands (1–1 mM). Panel C shows the long-term uptake of 50 μM AA in fibroblasts, measured by HPLC. DAA (D) and AA (E) uptake (1–1 mM) was also measured in plasma membrane-permeabilized control and ATS fibroblasts using the rapid filtration method and radiolabeled ligands (1–1 mM). Fibroblasts from P1, P3, P4, P5, P7, and P8 (panels A, B, D, E) or from P3, P4, P7, and P8 (panel C) were used. Results are expressed as means ± SEM of 6–10 experiments or (where SEM bars are missing) as means of two measurements.

controls and ATS patients. Decreased DAA (Fig. 3A) and unchanged AA transport (Fig. 3B) was observed in the patients' fibroblasts, indicating a DAA-specific failure of vitamin C transport. Investigation of long-term AA uptake and accumulation in the presence of physiological AA concentration (50 μM) revealed that the steady-state intracellular concentration of AA was set up at a lower level in patients' fibroblasts comparing with the controls (Fig. 3C).

To estimate the transport through endomembranes, the plasma membrane of the cells was selectively permeabilized. An even more dramatic decrease in DAA transport was found in cells from ATS patients under this condition, which indicated that the failure is due to a transporter resident in the endomembranes (Fig. 3D). AA transport was similar in permeabilized control and patient fibroblasts (Fig. 3E).

Re-expression of GLUT10 in ATS fibroblasts restores DAA transport

GLUT10 expression vector (pG10) and empty cloning vector (mock) were stably transfected in the skin fibroblasts of an ATS patient (P1). P1 was intentionally used in the re-expression experiments to avoid any interference with an eventual mutated protein present in the ATS fibroblasts, because of the almost complete lack of GLUT10 mRNA, due to the activation of
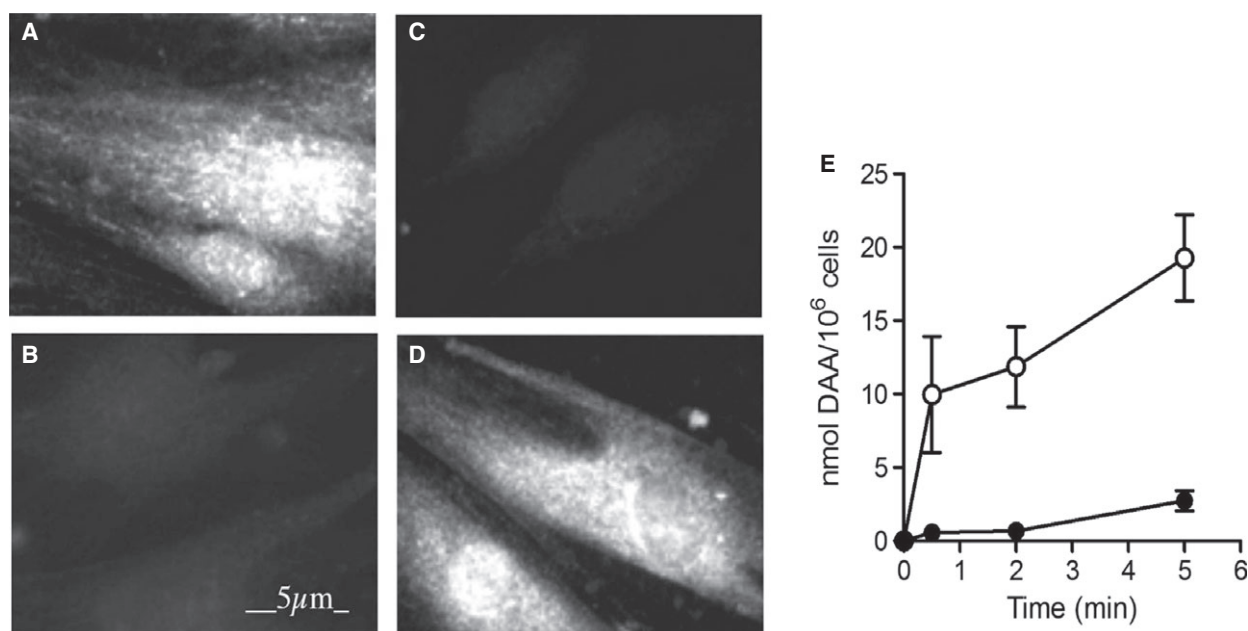
Fig. 4. Expression of GLUT10 in ATS fibroblasts restores DAA transport. Stable transfection of GLUT10 into the fibroblasts of the ATS patient P1 resulted in the appearance of GLUT10 protein in the cells, as it was verified by immunocytochemistry. A, control fibroblasts; B, ATS fibroblasts; C, ATS fibroblasts, mock transfected; D: pG10-transfected ATS fibroblasts. DAA (1 mM) transport activity could be measured in plasma membrane permeabilized, GLUT10 re-expressing cells (panel E; empty circles, means ± SEM of three experiments). In panel E, the DAA uptake in permeabilized ATS fibroblasts, shown in Fig. 3D, is also reported for comparison (black circles).

nonsense-mediated mRNA decay. These transfected cells do not only re-express GLUT10 but also rescue a control-like phenotype concerning the extracellular matrix homeostasis and GLUT10-dependent canonical signal transduction mechanisms, as shown in [25]. The absence of the immunoreactivity of GLUT10 was observed in nontransfected (Fig. 4B) and mock-transfected (Fig. 4C) cells. Re-expression of GLUT10 in patients' fibroblasts resulted in the appearance of GLUT10 immunoreactivity as revealed by immuno-cytofluorescence (Fig. 4D; for comparison see control fibroblasts, Fig. 4A). Re-expression also restored the impaired DAA transport activity in plasma membrane-permeabilized cells (Fig. 4E).

Discussion

Although mutations in the *SLC2A10* gene encoding the GLUT10 protein have been unequivocally identified as the genetic cause of ATS, the underlying pathogenetic mechanisms have not been elucidated up to now. The determination of the intracellular localization of GLUT10 and the identification of the ligand(s) it transports are crucial for further clarifying the role of GLUT10 in cardiovascular development. The results that we obtained demonstrate that GLUT10 is

a DAA transporter, and DAA transport is defective through the endomembranes of ATS fibroblasts.

We demonstrated, by incorporating *in vitro*-translated GLUT10 protein into liposomes, that it acts as a specific DAA/glucose transporter. Incorporation of microsomal proteins from control and ATS fibroblasts into liposomes showed that DAA transport was reduced in proteoliposomes containing patients' microsomal proteins. In plasma membrane-permeabilized cells a huge decrease in DAA transport of ATS patients' skin fibroblasts was observed, indicating the defect of DAA transport through the endomembranes. The deficient transport was restored by the re-expression of GLUT10 in ATS fibroblasts. Moreover, silencing of GLUT10 in hTERT fibroblasts also compromised DAA transport through the endomembranes.

The finding that GLUT10 acts as a DAA transporter is supported by some previous observations. The human phenotype of ATS could not be reproduced in GLUT10 mutant mice, as these mice showed minimal or no pathological signs [11,12]. The absence of a relevant phenotype in mice could be explained by the fact that mice, in contrast to humans, possess gulonolactone oxidase activity. Thus, mice are able to synthesize AA, independently of dietary intake.

It may be hypothesized that in AA-synthesizing species an enhanced AA production can compensate somehow for the reduced activity of organellar AA/DAA transporters. This hypothesis is reinforced by the relevant cardiovascular abnormalities that have been identified in a zebrafish ATS model [31]. This and other teleost species, like humans, lack a functional gulonolactone oxidase gene.

A glucose transporter in the endomembranes, especially in the ER has long been hypothesized as a component of the ER-localized glucose-6-phosphatase system [18]. The presence of GLUT10 in the endomembranes as a DAA and glucose transporter can be the molecular background of the functionally already observed transport activities [19,20,32]. However, it is probable that GLUT10 is not a single DAA/glucose transporter in the endomembranes. Functional studies indicated the heterogeneity of glucose transport in the ER [19,20]. Moreover, it was also demonstrated that various GLUT transporters can sufficiently mediate glucose transport en route from the ER to the plasma membrane [21]. These circumstances may provide an explanation for the fact that GLUT10 deficiency does not affect similarly all cell types.

The question arises: which function of DAA/AA is missing in the secretory compartment and/or nucleoplasm in ATS patients? Besides the general antioxidant role, AA acts also as a cofactor for 2-oxoglutarate/Fe^{2+}-dependent dioxygenases [33]. Prolyl- and lysyl-hydroxylases involved in the post-translational modification of collagen and other extracellular matrix proteins such as elastin belong to this group of the enzymes [34] as many nucleoplasmic DNA and histone demethylases [35]. DAA has been nominated as an electron acceptor in the process of oxidative protein folding, that is, in the formation of disulfide bonds [15,36]. Thus, shortage of AA in the nucleoplasm and in the lumenal compartments of the secretory pathway can depress the production of extracellular matrix proteins at both epigenetic and post-translational levels [22]. Indeed, changes of gene expression has been demonstrated by a recently published transcriptome analyses in ATS fibroblasts. The alterations affected the expression of several genes involved not only in TGFβ signaling and extracellular matrix organization and homeostasis but also in specific pathways that control cell energy balance and the oxidative stress response [25]. Although further studies are needed to clarify the role of these changes in the pathogenesis of ATS, the results presented here clearly show that GLUT10 can mediate the membrane transport of DAA and this transport is defective in the endomembranes of ATS fibroblasts.

Acknowledgements

Author contribution

CEN carried out subcellular fractionation and immunoblotting of the fractions, performed HPLC measurements, prepared proteoliposomes, contributed to transport measurements, performed the statistical analysis and drafted the manuscript. PM performed the experiments in hTERT fibroblasts. AG, RF and AB participated in the design of the study, contributed to transport measurements and immunocytochemistry. NZ, MR, NC and MC prepared and characterized human fibroblasts from ATS patients, re-expressed GLUT10, performed immunocytochemistry and participated in the design of the study, AW, BLC and PJC prepared and characterized human fibroblasts from controls and ATS patients, and participated in the design of the study, PG prepared the liposomes, SKN and TM carried out the in vitro translation of GLUT10, GB participated in the design of the study, performed transport measurements and analyzed the results, ÉM conceived of the study, participated in its design, coordinated the collaboration between the participant groups and wrote the manuscript. All authors reviewed the results and approved the final manuscript.

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