Arginase-flotillin interaction brings arginase to red blood cell membrane

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Abstract Flotillin-1 and arginase are both up-regulated in red blood cell membrane of type 2 diabetic patients. For studying why the soluble arginase can bind to the membrane and whether such binding would modify arginase activity, the arginase1 and related proteins were cloned and expressed. The results showed that flotillin-1 can interact with arginase1, and hence arginase activity was up-regulated by 26.8%. It was estimated that about 61% of arginase1 is bound to the membrane mediated by flotillin-1. The arginase activity in diabetic patients was significantly higher than that of the controls (752.4 ± 38.5 U/mg protein vs 486.7 ± 28.7 U/mg protein).

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1. Introduction

Current therapeutic approaches of type 2 diabetes were largely developed in the absence of defined molecular target or even solid understanding of disease pathogenesis [1,2]. The blood should be regarded as the primary target tissue in diabetes mellitus. It is reasonable to presume that the properties of red blood cell (RBC) membrane must suffer variation during disease development [3]. Our previous studies have provided evidence that flotillin-1 and arginase1 proteins were both up-regulated in RBC membranes of type 2 diabetic patients [4].

Flotillins consist of two family members: flotillin-1 (reggie-2) and flotillin-2 (reggie-1) [5]. Flotillin-1, flotillin-2 and stomatin are the most abundant membrane proteins in RBC lipid rafts. They present as independently organized high-order oligomers and act as separate scaffolding components at the cytoplasmic face of RBC lipid rafts [6], which have been implicated in numerous cellular processes including signal transduction and membrane trafficking. Flotillin-1 plays an important role in the second signaling pathway required for insulin-stimulated glucose transport [7].

Arginase, the final enzyme in the urea cycle, is responsible for the hydrolysis of L-arginine to urea and L-ornithine. Arginase has two forms, designated arginase1 and arginase2, and arginase1 is present in the high concentration in RBC. Arginase shares a common substrate, L-arginine, with nitric oxide synthase (NOS), thus increased expression of arginase in diabetic human cavernosal tissue may down-regulate NO production by competition with NOS [8]. It has been known that NOS is involved in insulin action [9]. NOS was also found in the human RBC [10,11]. The expression of arginase1 in RBC membranes is also high in patients, which confirmed that NO and arginase pathways are involved in the development of human type 2 diabetes [4]. Arginase activity is elevated in livers of diabetic animals [12]. But up to now there is no information available about why the soluble arginase is located on cell membrane and how its enzymatic activity is regulated in RBC. In present study, the cytoplasmic domain of band 3 (cd3), arginase, flotillin-1, and related proteins were cloned and expressed in Escherichia coli for searching the partner in association with arginase, and for studying the effect of such protein–protein interaction on the arginase activity.

2. Materials and methods

2.1. Cloning, expressing and purifying GST-arginase1, His6-arginase1, His6-cdb3, His6-flotillin1 and His6-flotillin2

For recombinant arginase1 with glutathione S-transferase (GST) or hexahistidine (His6) tag, we used human embryo liver cDNA as nested PCR reaction’s template, which was kindly provided by Dr. Ping Xu, Laboratory of Genomic Physiology, School of Life Sciences, Fudan University. The first round primers were 5′-TGGAGGTGTAGT-GACTGG-3′ and 5′-CAAGAGGGGTTGAGGAGG-3′. The second round primers used were 5′-ATGAATCCTGACCGCCAAGTCCACC-3′ and 5′-AATCGTGGATATTAGTGGGTCCT-3′ (the underlined base pairs represent the sequences digested by restriction endonuclease EcoRI and XhoI respectively). The product of second round PCR reaction was double digested by EcoRI and XhoI, ligated with EcoRI and XhoI double digested pGEX-4T-1. The positive clone was verified by DNA sequencing. The His6-arginase1 was constructed by double digested arginase1 fragment ligated with double digested pET21a containing both N-terminal and C-terminal His6 tag, which was constructed from plasmid pET21a in our laboratory.GST-arginase1 and His6-arginase1 were expressed and purified as Pharmacia’s GST and His tag fusion protein protocols, respectively.

The recombinant fusion protein His6-cdb3, His6-flotillin1 and His6-flotillin2 with hexahistidine tag in the C-terminal were constructed and purified as described previously [13,14].

2.2. Preparation of membrane and cytoplasm fractions of RBC

Freshly drawn blood samples from 6 patients with Chinese type 2 diabetes were obtained from the Shanghai Changhai Hospital. Subjects were 42–61 years old with a fasting glucose level of (10.8 ± 1.8) mM and 5 fresh blood samples from healthy individuals were obtained from the Shanghai Red-Cross Blood Center. RBC membranes were prepared by hypotonic lysis of RBC in 5 mM sodium phosphate (pH 7.4). After hemolysis the membrane and cytoplasm fractions were separated by centrifugation. Protein concentration was calibrated using BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA).

2.3. Far-Western blot

The recombinant proteins were electrophoresed on SDS-PAGE, and then transferred onto nitrocellulose membrane. The nitrocellulose
was blocked with blocking buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.05% (v/v) Tween 20, and 5% (w/v) low fat milk powder) overnight at 4 °C. The nitrocellulose was then incubated with 100 μg of a probe protein added to the binding buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.0, and 1% (w/v) low fat milk powder) for 4 h at room temperature. After washing, the membrane was incubated with anti-band 3 (Sigma Inc., St. Louis, MO, USA) or anti-flotillin monoclonal antibody (BD Biosciences, San Jose, CA, USA) and secondary antibody together for 1 h at 37 °C. Finally, membrane-bound antibodies were detected using SuperSignal WestPico (Pierce Biotechnology, Rockford, IL, USA) chemiluminescent substrate solution for 1 min and then pressed against an autoradiographic film.

2.4. Pull-down assay
The purified recombinant GST-arginase1 or control GST protein was loaded on the MagneGST glutathione particles (20 μl) equilibrated with buffer A (PBS containing 0.1% (v/v) Triton X-100) and blocked by bovine serum albumin (100 μg). One hundred microgram of the probe protein (His6-cdb3, His6-flotillin-1 or His6-flotillin-2) was loaded on the particles. The mixture was shaken gently for 5 h at 4 °C. After washing with PBS containing 0.1% (v/v) Triton X-100, the proteins were analyzed by SDS-PAGE followed with Western blot as described previously [13].

2.5. Assay of arginase1 activity
The arginase activity of recombinant His6-arginase1 or arginase in RBC was determined with standard procedures for the detection of urea by reaction with diacetyl monoxime [15]. Briefly, 50 μl of samples was added to a reaction buffer (0.1 mM Tris–HCl buffer, pH 7.4) containing 0.2 M L-arginine and 0.5 μM MnCl2 and incubated for 60 min at 37 °C followed by stopping reaction by addition of 1 M HCl. Then the concentration of urea was measured. The arginase activity was calculated and expressed in units per mg of proteins. One unit of arginase activity is equivalent to the conversion of 1 μmol urea/h at 37 °C.

3. Results

3.1. Expression and purification of GST-arginase1, His6-arginase1, His6-cdb3, His6-flotillin-1 and His6-flotillin-2
The expression of soluble fraction of the recombinant protein flotillin-1 or flotillin-2 with GST tag or thioredoxin tag in the N-terminal or His6 tag in C-terminal was quite low (data not shown). So we choose His6 tag to express and purify the recombinant proteins for the reason of reducing the effect of large fusion tag [14].

The solubility of the fusion protein arginase1 with GST or His6 tag was both high. The fusion proteins of the desired molecular weight, approximately 37-kDa for His6-arginase1 and 63-kDa for GST-arginase1, were expressed. GST-arginase1 and His6-arginase1 were purified by MagneGST glutathione particles and Ni-NTA column, respectively (Fig. 1).

3.2. Interactions among flotillin-1, flotillin-2, cdb3 and arginase1
A series of Far-Western blot assay with recombinant proteins His6-flotillin-1, His6-flotillin-2 and His6-cdb3 were performed, respectively. There was interaction between flotillin-1 and flotillin-2 (Fig. 2A and B). Using pull-down assay the association of GST-arginase1 with His6-flotillin-1 was identified (Fig. 2C). We also tried to study the association of GST-arginase1 with His6-flotillin-1 by Far-Western assay, but significant interaction was not observed (data not shown). This infers that the proper conformation of the proteins would be necessary for interaction between arginase1 and flotillin-1.

3.3. Modulation of arginase1 activity by flotillin-1
What would be the physiological role of flotillin-arginase interaction in RBC? Fig. 3A shows that the enzymatic activity of His6-arginase1 increased significantly after interaction with His6-flotillin-1, meanwhile cdb3 or flotillin-2 has no modulation effect on arginase activity. The modulation of arginase1 activity by flotillin-1 appears a dose dependence manner as shown in Fig. 3B. The arginase1 activity can be up-regulated by 26.8%.

3.4. Different arginase activity in cytoplasm and membrane of RBC
Our previous work indicated that the protein spot of soluble arginase always appeared in the two-dimensional gel of RBC membranes [4]. So it would be interesting to compare the enzymatic activities in both membrane and cytoplasm circumstances for the purpose of identifying the location of arginase in RBC. Table 1 shows the enzymatic levels of arginase in cytoplasm and membrane of RBC, respectively. The result infers that quite a lot of arginase would be bound on the membrane of RBC.

3.5. Comparison of arginase activity between RBC membranes from type 2 diabetic patients and healthy controls
The enzymatic levels of arginase from 6 diabetic patients and 5 healthy subjects were measured. Arginase activity (means ± S.D.) of RBC membrane from diabetic patients and healthy controls is 752.4 ± 38.5 U/mg and 486.7 ± 22.6 U/mg, respectively. That is, the arginase activity in RBC membrane was increased by about 54.6% (P < 0.05, compared to healthy controls, Student’s t-test) in diabetic patients.

4. Discussion

Human flotillin-1 is a specific hydrophobic protein of biomembrane microdomain lipid rafts, which exerts important process in the pathway of insulin-stimulated translocation of glucose transporter 4 in adipose and muscle cells [16,17]. Previous studies have proved that flotillin-1 was up-regulated in RBC membrane of type 2 diabetic patients [4]. But we still...
did not know the detailed relationship of flotillin-1 and type 2 diabetes in molecular level. It has been reported that flotillins 1 and 2 form a high-order oligomeric complex with stomatin in RBC lipid rafts [6]. In accordance with this report, we did find the direct interaction between flotillin-1 and flotillin-2 (Fig. 2A and B), moreover, our present study also identified the interaction between flotillin-1 and arginase1 (Fig. 2C). Out of our previous presumption [4], it was not found that there would be the interaction of arginase1 with cd3. Cd3, acts as an attachment site for RBC skeleton by binding ankyrin, protein 4.1 and protein 4.2, helping to maintain the mechanical properties and integrity of the cell. It was well demonstrated that the ability of band 3 to anchor the membrane skeleton to the lipid bilayer is one of the essential factors to contribute to the deformability and stability of the human RBC membrane using ektacytometer and micropipette aspiration technique [18]. Besides, cd3 also interacts with several glycolytic enzymes, p72(syk) protein tyrosine kinase, hemoglobin and hemichromes to take part in regulating the metabolic activity and sensing the aged or abnormal RBCs [19,20]. So it would be natural to make a similar suggestion that the display of soluble arginase in the two-dimensional gel of the membrane

![Fig. 2. Association among flotillin-1, flotillin-2, cd3 and arginase1. Fusion proteins were run on SDS–PAGE and subjected to Far-Western blot (A and B) or pull-down assay (C). (A) His6-F1 (positive control), His6-F2 and His6-cd3 (each 15 μg) were transferred onto nitrocellulose membrane, and His6-F1 (100 μg) was added (lane 2 and 3) as a probe protein using anti-flotillin-1 monoclonal antibody. (B) His6-F2 (positive control), His6-F1 and His6-cd3 (each 15 μg) were transferred to nitrocellulose membrane, probe protein His6-F2 was added (lane 5 and 6), detected by anti-flotillin-2 monoclonal antibody. (C) Purified GST (negative control) and GST-ARG were loaded on the MagneGST glutathione particles balanced with buffer A (PBS containing 0.1% (v/v) Triton X-100). The target proteins of purified recombinant His6-cd3, His6-F1 and His6-F2 were loaded on the resin with gentle shaking 4 h at 4 °C. After washing, the proteins eluted were analyzed by SDS–PAGE followed by Western-blot using anti-band 3, anti-flotillin-1 and anti-flotillin-2 monoclonal antibodies, respectively. F1 and F2 represent flotillin-1 and flotillin-2, respectively. ARG represents arginase.]

![Fig. 3. The effect of interaction between arginase1 and flotillin-1 on arginase activity. (A) The excessive amount of His6-flotillin-1, His6-flotillin-2 and His6-cd3 was incubated with His6-arginase1 for 4 h at 37 °C, respectively, followed by adding to reaction buffer for 60 min at 37 °C. The urea concentration in each sample was then measured. The arginase1 activity was (2.11 ± 0.31) × 10^5 U/mg (control), (2.68 ± 0.08) × 10^5 U/mg (ARG + F1), (2.16 ± 0.12) × 10^5 U/mg (ARG + F2) and (2.20 ± 0.15) × 10^5 U/mg (ARG + cd3). Data were from six independent experiments (means ± S.D., *P < 0.05 compared to control, Student’s t-test). (B) Dose dependence of the effect of flotillin-1 on arginase 1 activity. His6-arginase1 (1 mg) was incubated with different amount of flotillin-1 (0–6.0 mg) in 5 ml of PBS (pH 7.4) for 4 h at 37 °C, then 100 μl of each sample was added to the reaction buffer for 60 min at 37 °C. The urea concentration in each sample was then measured. Data were from three independent experiments (means ± S.D.). F1 and F2 represent His6-flotillin-1 and His6-flotillin-2, respectively. ARG represents His6-arginase1.]

![Table 1: Arginase1 activities in the membranes and cytoplasm of RBC.](attachment:image)

*RBCs were hemolyzed in five volumes of 5 mM sodium phosphate (pH 7.4), and the cytoplasm and membranes were separated by centrifugation at 20,000 ×g. The enzymatic activities of both membranes and cytoplasm fractions were assayed. The arginase activities in the membranes and cytoplasm were calculated according to the total proteins in the membranes and cytoplasm, respectively. Protein concentration was calibrated using BCA protein assay kit.*

*Data were from five independent experiments (means ± S.D.).*
fraction would be due to its interaction with cdb3. But this is not the case according to the present study. In addition, the direct association of arginase1 with glucose transporter 1, another major membrane protein in the RBC membrane, was also not observed (data not shown). The interaction of soluble enzyme arginase with flotillin-1 would be flotillin-1’s another role. In other words, arginase pathways and glucose transporter translocation would be linked together to involve in the development of human type 2 diabetes.

Fig. 3A shows that the activity of the recombinant His6-arginase1 was (2.11 ± 0.31) × 10^5 U/mg protein, which is much higher than that of RBC membranes (486.7 ± 28.7 U/mg total protein) (Table 1). This is obvious because the recombinant His6-arginase1 was purified protein, meanwhile the arginase activity of RBC membranes was calculated according to the total proteins on the membranes. The reason is similar for big difference of the arginase activity between membranes and cytoplasm fractions of RBC (Table 1) because of the different ratio of amount of arginase in membrane and cytoplasm. But the interesting point is that the significant arginase activity was found in RBC membranes, which demonstrated again that soluble arginase can bind to the RBC membrane in vivo. The value of membrane-bound fraction of the arginase1 can be roughly estimated. For human RBC, the average amount of hemoglobin in a RBC is 25–35 pg, and the total amount of membrane proteins of a RBC is 0.57 pg [21]. As a first approximate, the enzymatic activity of arginase1 on the membrane is assumed to be 1.28-fold higher than in cytoplasm (Fig. 3). Based on these parameters and the data of Table 1, it can be calculated that about 61% of arginase1 in RBC is bound to the membrane. To eliminate the tag effect, the recombinant His6-arginase1 was used to determine the arginase activity (Fig. 3). But in fact the enzymatic activity of GST-arginase1 was similar to that of His6-arginase1, and this means that there was no significant functional difference between His6-arginase1 and GST-arginase (data not shown).

Arginase is an enzyme that shares a common substrate with NOS, thus arginase may down-regulate NO production by competing with NOS for the substrate, L-arginine [8]. Alterant arginase is implicated in the pathophysiology of many symptoms, including diabetic disease specially [8,12]. However, the molecular mechanism that leads to the elevation of the arginase activity in diabetes is still not known. The present studies, for the first time, show that the soluble arginase can locate on the membrane in RBC through its association with membrane protein flotillin-1, and hence its enzymatic activity is up-regulated. The result also indicates that this would be another way to connect the membrane and the cytosol in RBC besides of cdb3. This conclusion is further confirmed by the experiment of dose dependence of the effect of flotillin-1 on arginase1 activity (Fig. 3B).

In conclusion, the present investigation shows that arginase can bind to the membrane through its association with flotillin-1 but not via cdb3. Such interaction can up-regulate arginase activity. The abnormality of arginase activity in RBC membrane of diabetic patients may play an important role in the development of human type 2 diabetes.

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