Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 309 (2003) 196–200
BBRC
www.elsevier.com/locate/ybbrc

Protein disregulation in red blood cell membranes of type 2 diabetic patients

Ming Jiang,[a] Linzhi Jia,[a] Weihua Jiang,[a] Xiaojian Hu,[a] Hanqing Zhou,[a] Xin Gao,[b] Zhiqiang Lu,[b] and Zhihong Zhang[a,*]

[a] *Department of Physiology and Biophysics, School of Life Sciences, Fudan University, Shanghai 200433, China*
[b] *Department of Endocrinology, Zhongshan Hospital, Fudan University, Shanghai 200433, China*

Received 28 July 2003

Abstract

The proteomics analysis was used to search for the membrane proteins related to the type 2 diabetes in human red blood cell (RBC). To improve the solubilization and separation for membrane proteins during two-dimensional electrophoresis (2-DE), several types of chaotropes and surfactants were tested. The optimized condition was then screened. About 1000 protein spots from RBC membranes can be resolved on the 2-D gel. To compare the 2-DE patterns between RBC membranes of type 2 diabetic patients and healthy controls, a total of 42 proteins that were differentially expressed were found. The analysis shows that flotillin-1, a recently discovered membrane protein of RBC lipid rafts, appears to be affected in the disease. The result would be quite interesting because flotillin-1 in adipocytes functions is related to stimulate activation of glucose transporter 4 in response to insulin. Additionally, syntaxin 1C and arginase were also disregulated in patient RBC membranes.

Keywords: Type 2 diabetes; RBC membranes; Lipid rafts; Poteomics; Flotillin; Syntaxin; Arginase

Type 2 diabetes mellitus accounts for 90–95% of all diabetes. Current therapeutic approaches were largely developed in the absence of defined molecular target or even solid understanding of disease pathogenesis [1,2].We have observed that the rate of glucose entry into red blood cell (RBC) was decreased significantly in type 2 diabetic patients compared with healthy controls and this was due to the structural change of glucose transporter 1 (Glut 1) [3]. There is substantial interest in the identification of more membrane proteins related to the disease development using proteomics. Because hydrophobic membrane proteins simply do not dissolve in traditional solvents used for isoelectric focusing (IEF), the first step of two-dimensional electrophoresis (2-DE), so classical techniques used in 2-DE are of poor efficiency for their analysis [4].

In this study, several types of surfactants were used for optimizing the condition of solubilization for RBC membrane proteins during isoelectric focusing. A total of 42 protein spots on the 2-D gel were expressed differentially between RBC membranes of type 2 diabetic patients and the healthy controls. Among them flotillin-1, a recently discovered membrane protein of RBC lipid rafts [5], syntaxin 1C, and arginase appear to be affected in the disease.

Materials and methods

Sample preparation. Freshly drawn blood samples from 8 patients with Chinese type 2 diabetes were obtained from the Zhongshan Hospital in Shanghai. Subjects were 48–65 years old with a fasting glucose level of (11.6 ± 1.9) mM. The 12 fresh healthy blood samples were obtained from the Shanghai Red Cross Blood Center or from healthy volunteers. RBC membranes were prepared by hyposmotic lysis of RBC in 5 mM sodium phosphate (pH 7.4). After washing several times with PBS containing 155 mM NaCl and 5 mM sodium phosphate (pH 7.4), the membrane fraction was collected. Protein

* Corresponding author. Fax: +86-21-6565-0149.
E-mail address: zhzhang@fudan.edu.cn (Z. Zhang).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-291X(03)01559-6

concentration was calibrated using BCA protein assay reagent kit (Pierce). The resulting pellet was freeze-dried before storage at −20 °C.

Electrophoresis. Two-dimensional electrophoresis using immobilized pH gradient (IPG) strips was performed as described [6]. RBC membrane samples containing 100 μg proteins were dissolved into 300 μl rehydration buffer containing tested surfactant. The solution was applied onto the IPG strips (pH 3–10, 17 cm, Bio-Rad). IEF was performed after rehydration of the IPG strips. The gel strips were then equilibrated for 2×15 min with gentle shaking in 8 ml equilibration solution containing 50 mM Tris–HCl, 6 M urea, 30% glycerol, and 2% SDS. DTT (1% w/v) was added to the first and iodoacetamide (2.5% w/v) to the second. SDS polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using homogenous 10% polyacrylamide gels. After electrophoresis, gels were fixed followed by staining with silver nitrate using silver stain kit (Bio-Rad) for the next image analysis. For further protein identification with mass spectrometry, gels were stained with Coomassie brilliant blue R-250.

For 1-D electrophoresis, protein samples containing 20 μg proteins were dissolved by sample buffer containing 2% SDS, 50 mM Tris–HCl, 10% glycerol, 50 mM DTT, and a trace of bromophenol blue. Gel electrophoresis was performed in 10% polyacrylamide gels in an Xcell Mini-Cell electrophoresis system (Bio-Rad).

Image analysis. Following capture of the images in digital format and editing of 2-D gel images by Molecular Imaging FX (Bio-Rad), image analysis was performed using the PDQuest software V.7.0 according to the protocol provided by the manufacturer. Matching of the spots was performed by use of a reference gel prepared from four gels. Spot standardization was carried out for all matched spots. Spot volume (intensity) was then normalized as percentage of the total spot volume using spots that were present in all gels.

Identification of proteins. The protein spot in 2-D gel was excised and destained. After in-gel tryptic digestion, the peptide mass fingerprinting was analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE PRO). The results of peptide mass fingerprinting were identified using the programs MS-Fit (http://www.prospector.ucsf.edu) or Mascot (http://www.matrixscience.com). Additionally, for confirming the identification by mass spectrometry, Western blotting was performed in the same protein spot on the gel using monoclonal antibody against flotillin-1 (Transduction Laboratories) with subsequent detection by horseradish peroxidase-linked goat anti-mouse IgG (ImmuclubLabs) and the SuperSignal West Pico chemiluminescent kit (Pierce). Western blotting using monoclonal antibodies against flotillin-1 and β-actin was also carried out on the traditional 1-D SDS–PAGE for confirming disregulated expression of flotillin-1 in RBC membranes of type 2 diabetic patients.

Results

In recent years, a greater understanding of the biological and pharmacological importance of membrane proteins has prompted significant efforts to improve the separation of these less soluble proteins using in proteomics analysis with 2-DE [7,8]. According to the most recent developments in the area of membrane protein solubilization, we have tested several types of surfactants including CHAPS, SB3-10, SB3-14, ASB3-16, Triton X-114, and ASB14 (tetradecanoylamido propyl dimethyl ammonio propane sulfonate, CN Biosciences). Among these surfactants, ASB14 has been used successfully to solubilize integral membrane proteins from *Escherichia coli*, *Arabidopsis*, and RBCs [4,7]. Band 3 is the most abundant integral membrane protein in RBC. So band 3 was chosen as the model protein to evaluate the solubility problems frequently encountered in the analysis of membrane proteins by 2-DE [4]. First, we tested the efficiency of membrane protein extraction of the surfactants according to the 1-D SDS–PAGE. Although SDS is the most effective surfactant of dissolving membrane protein (Fig. 1), but because of its electric charge SDS is restricted to use at IEF. It is quite clear from Fig. 1 that ASB14 can extract more band 3 from RBC membranes than CHAPS which is usually used in IEF step. Sample buffer containing 6 M urea, 2 M thiourea, 20 mM DTT, 0.2% Bio-Lyte, and 2% ASB14 was finally screened for solubilization of RBC membrane proteins. As shown in Fig. 2, 2% ASB14 in the extraction buffer during IEF can dissolve band 3 proteins shown by ovals on gels more effective than 4% traditional CHAPS, and we were able to resolve, detect, and quantify as many as 1000 protein spots on the 2-D gels from RBC membranes.

Based on the comparison of 2-DE patterns between RBC membranes of type 2 diabetic patients and the healthy controls, a total of 42 protein spots that were highly disregulated ($P < 0.05$) in diabetes samples (Fig. 3) were found. Among them 27 spots showed up-regulation and 15 spots showed down-regulation in RBC membranes of the patients. The protein spot 11 in

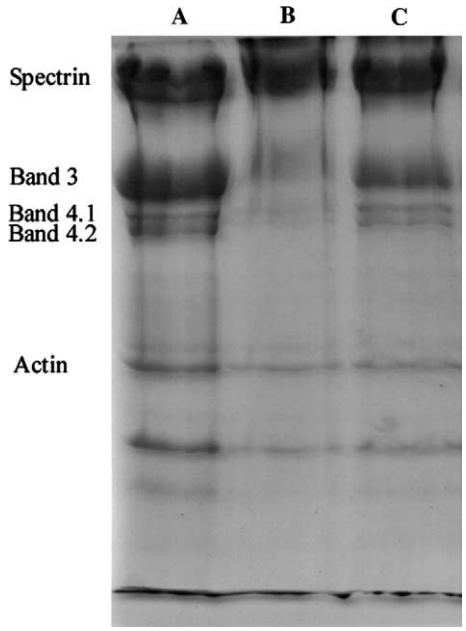

Fig. 1. Sodium dodecylsulfate–polyacrylamide gel electrophoresis patterns of 20 μg ghost proteins after extraction by sample buffer containing 2% SDS (A), 2% CHAPS (B), and 0.5% ASB14 (C), respectively. The amount of band 3, the most abundant integral membrane protein in RBC, can be used to evaluate the efficiency of membrane protein extraction of the surfactants. Spectrin, band 4.1, band 4.2, and actin are the peripheral membrane proteins in RBC membranes.

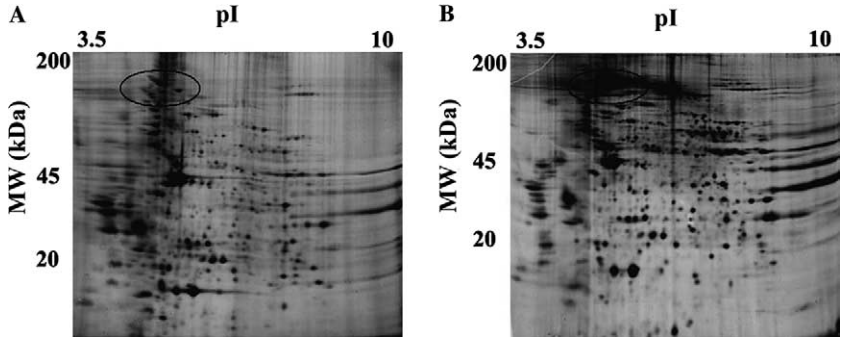

Fig. 2. Two-dimensional gels of proteins from RBC membranes using 4% CHAPS (A) or 2% ASB14 (B) as surfactant for protein solubilization. A total of 100 μg proteins of RBC membranes were dissolved into 300 μl rehydration buffer containing 4% CHAPS (A) or 2% ASB14 (B) as surfactant. The solution was applied onto 17 cm IPG (pH 3–10) strips. Rehydration was programmed to be carried out at 20 °C for 14 h followed by IEF step. SDS–PAGE was carried out using homogenous 10% polyacrylamide gels. After electrophoresis, gels were fixed and stained with silver nitrate. The marks of ovals on gels are the position of band 3.

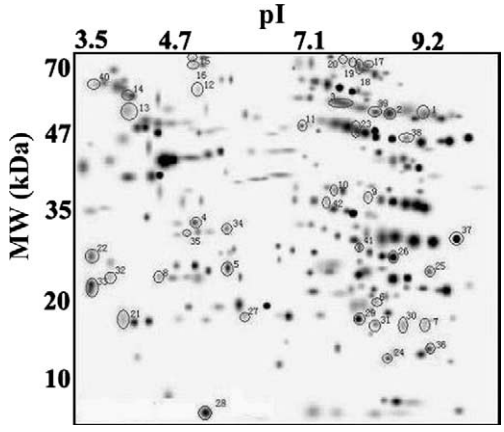

Fig. 3. Disregulated proteins on gels from RBC membranes of type 2 diabetic patients. There were a total of 42 proteins (labeled spots) that were highly disregulated ($P < 0.05$) in diabetes samples on 2-D gels from RBC membranes. Of them spot 11 was identified as flotillin-1 (MW 47.3 kDa, p*I* 7.1).

the Fig. 3 was identified as flotillin-1 with peptide mass fingerprinting using MALDI-TOF mass spectrometer. The identification of flotillin-1 was furthermore confirmed using Western blotting in the same spot position on the gel. By image analysis, the expression of flotillin-1 in 8 patients suffering from type 2 diabetes was increased by (4.8 ± 0.2) times ($P < 0.05$) compared with 12 healthy controls. Western blotting on the traditional 1-D SDS–PAGE of the proteins in RBC membranes also confirmed this (Fig. 4).

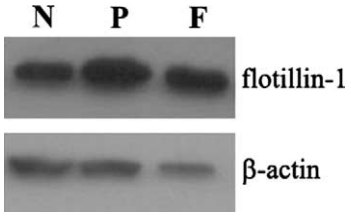

Fig. 4. Western blotting using monoclonal antibodies against flotillin-1 and β-actin on the traditional 1-D SDS–PAGE of RBC membrane proteins from diabetic patients (P) and the healthy control (N). Flotillin-1 and β-actin proteins (F) were used as the positive controls, which were subjected to immunoblotting with the corresponding antibodies.

The protein spot 8 was identified as syntaxin 1C and its expression in patient RBC membranes was decreased by (8.3 ± 0.1) times ($P < 0.01$) compared with the healthy controls. The protein spot 42 was identified as arginase and its expression in patient was up-regulated by (37.2 ± 3.7) times ($P < 0.001$) compared with the healthy controls.

Discussion

The blood vessel should be regarded as the primary target organ in diabetes mellitus. It is reasonable to presume that the properties of RBC, especially plasma membrane, must suffer variation during disease development [3,9–11]. The present study showed, for the first time, that flotillin-1 protein was affected in type 2 diabetes RBC membranes. Flotillin-1 protein was first identified in a screen of mouse adipose tissue for novel marker of lipid rafts/caveolae [12]. It is enriched in the Triton X-100 insoluble buoyant fraction after sucrose density centrifugation, which is indicative of their association with lipid rafts microdomains in the membranes. Recently, it was reported that flotillins are the most abundant raft proteins in human RBC [5]. It is now clear that these lipid rafts microdomains act as platforms for conducting a variety of cellular functions, such as vesicular trafficking and signal transduction [13–16]. Our result shows that flotillin-1 in RBC membranes appears to be affected in the type 2 diabetes. Although we do not know yet the relationship between flotillin-1 and the disease of type 2 diabetes, but it is quite clear now about the function of flotillin-1 in translocation of glucose transporter Glut 4. In muscle and adipose tissue, flotillin-1 functions as an adaptor protein that recruits a signaling complex of proto-oncoprotein

c-Cbl/c-Cbl-associated protein to lipid rafts leading to Glut 4 translocation to the plasma membranes in response to insulin [17–19].

Translocation of Glut 4 to the plasma membranes in muscle and adipose tissue is mediated by vesicle transport, vesicle docking, and membrane fusion. Intense research has led to the general belief that all types of intracellular membrane fusions share a common mechanism. Syntaxin 1 on the target membrane binds to SNAP-25 and vesicular synaptobrevin/VAMP to assemble the core complex of the membrane fusion machine [20,21]. Decreased expression of syntaxin 1C in patient 2D-gel infers that translocation of Glut 4 to the plasma membranes would be impaired, even if the expression of flotillin 1 is compensatory increased. On the other hand, impaired membrane fusion machine must affect exocytosis of insulin secretory granule in the pancreatic β-cell. Actually, it was observed that protein levels of syntaxin 1 and SNAP-25 in islets isolated from GK rat, a nonobese rodent model of type 2 diabetes, decreased to approximately 60% of the levels in control rat islets, and hence insulin secretion was impaired [22].

Nitric oxide (NO) is a kind of second messengers, which is synthesized by nitric oxide synthase (NOS). It has been known that NOS is involved in insulin action [23]. It is also well documented that the major causative factor contributing to dysfunction of penile erection in diabetic patients is the impaired NO synthesis. Arginase is an enzyme that breaks down arginine. It shares a common substrate, L-arginine, with NOS, thus increased expression of arginase in diabetic human cavernosal tissue may downregulate NO production by competition with NOS [24]. Our result demonstrated that the expression of arginase in RBC membranes is also very high (about 37-fold) in patients, which shows that NO and arginase pathways are involved in the development of human type 2 diabetes. A lot of glycolytic enzymes in RBC are interacted with the cytoplasmic domain of band 3 (cdb3) and hence the function of these enzymes would be regulated [25]. We can make a similar suggestion that the display of soluble arginase in the 2-D gel of the membrane fraction would be due to its interaction with cdb3.

Further studies will have to unravel the detailed pictures of changes in the protein-protein interaction networks through flotillin-1, syntaxin 1C, and arginase, respectively, in RBC and adipocytes of type 2 diabetes. Most importantly, it will be critical to screen the 42 disregulated proteins for evaluation of some as "disease-specific proteins" for easy diagnosis using RBC or as the potential targets for drug discovery.

Acknowledgment

This work was supported by Grant 2001CB5102 from the National Basic Research Priorities Program of China.

References

[1] D.E. Moller, New drug targets for type 2 diabetes and the metabolic syndrome, Nature 414 (2001) 821–827.
[2] A.R. Saltiel, New perspectives into the molecular pathogenesis and treatment of type 2 diabetes, Cell 104 (2001) 517–529.
[3] X.J. Hu, F. Peng, H.Q. Zhou, Z.H. Zhang, W.Y. Cheng, H.F. Feng, The abnormality of glucose transporter in the erythrocyte membrane of Chinese type 2 diabetic patients, Biochim. Biophys. Acta 1466 (2000) 306–314.
[4] T. Rabilloud, T. Blisnick, M. Helle, S. Luche, R. Aebersold, J. Lunardi, C. Braun Breton, Analysis of membrane proteins by two-dimensional electrophoresis: comparison of the proteins extracted from normal or *Plasmodium falciparum*-infected erythrocyte ghosts, Electrophoresis 20 (1999) 3603–3610.
[5] U. Salzer, R. Prohaska, Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts, Blood 97 (2001) 1141–1143.
[6] A. Gorg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, The current state of two-dimensional electrophoresis with immobilized pH gradients, Electrophoresis 21 (2000) 1037–1053.
[7] M.P. Molloy, Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients, Anal. Biochem. 280 (2000) 1–10.
[8] V. Santoni, M. Molloy, T. Rabilloud, Membrane proteins and proteomics: Un amour impossible?, Electrophoresis 21 (2000) 1054–1070.
[9] B. Chong-Martinez, T.A. Buchanan, R.B. Wenby, H.J. Meiselman, Decreased red blood cell aggregation subsequent to improved glycaemic control in Type 2 diabetes mellitus, Diabetic Med. 20 (2003) 301–306.
[10] N. Kawamura, T. Ookawara, K. Suzuki, K. Konishi, M. Mino, N. Taniguchi, Increased glycated Cu,Zn-superoxide dismutase levels in erythrocytes of patients with insulin-dependent diabetis mellitus, J. Clin. Endocrinol. Metab. 74 (1992) 1352–1354.
[11] F. Marques, M.E. Crespo, Z.I. Silva, M. Bicho, Insulin and high glucose modulation of phosphatase and reductase enzymes in the human erythrocytes: a comparative analysis in normal and diabetic states, Diabetes Res. Clin. Pract. 47 (2000) 191–198.
[12] P.E. Bickel, P.E. Scherer, J.E. Schnitzer, P. Oh, M.P. Lisanti, H.F. Lodish, Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins, J. Biol. Chem. 272 (1997) 13793–13802.
[13] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[14] F. Galbiati, B. Razani, M.P. Lisanti, Emerging themes in lipid rafts and caveolae, Cell 106 (2001) 403–411.
[15] K. Simons, D. Toomere, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–41.
[16] D. Kalka, C. von Reitzenstein, J. Kopitz, M. Cantz, The plasma membrane ganglioside sialidase cofractionates with markers of lipid rafts, Biochem. Biophys. Res. Commun. 283 (2001) 989–993.
[17] N.J. Bryant, R. Govers, D.E. James, Regulated transport of the glucose transporter Glut 4, Nat. Rev. Mol. Cell Biol. 3 (2002) 267–277.
[18] P.H. Ducluzeau, L.M. Fletcher, H. Vidal, M. Laville, J.M. Tavare, Molecular mechanisms of insulin-stimulated glucose uptake in adipocytes, Diabetes Metab. 28 (2002) 85–92.
[19] C.A. Baumann, A.R. Saltiel, Spatial compartmentalization of signal transduction in insulin action, BioEssays 23 (2001) 215–222.
[20] D. Fasshauer, W.K. Eliason, A.T. Brunger, R. Jahn, Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly, Biochemistry 37 (1998) 10354–10362.

[21] K.M.S. Misura, R.H. Scheller, W.I. Weis, Three-dimensional structure of the neuronal-Sec 1-syntaxin 1a complex, Nature 404 (2000) 355–362.
[22] S. Nagamatsu, Y. Nakamichi, C. Yamamura, S. Matsushima, T. Watanabe, S. Ozawa, H. Furukawa, H. Ishida, Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion, Diabetes 48 (1999) 2367–2373.
[23] R.R. Shankar, Y. Wu, H.Q. Shen, J.S. Zhu, A.D. Baron, Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance, Diabetes 49 (2000) 684–687.
[24] T.J. Bivalacqua, W.J. Hellstrom, P.J. Kadwitz, H.C. Chapion, Increased expression of arginase II in human diabetic corpus cavernosum: in diabetic-associated crectile dysfunction, Biochem. Biophys. Res. Commun. 283 (2001) 923–927.
[25] P.S. Low, Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions, Biochim. Biophys. Acta 864 (1986) 145–167.