Original Article

RhoA Distribution in Renal Caveolar Fractions in Experimental Type 1 Diabetes

(caveolin-1 / co-immunoprecipitation / kidney / RhoA / sucrose fractionation / type 1 diabetes)

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Abstract. Caveolae act as signalling platforms serving as concentrating points for numerous signalling molecules, as well as regulating flux through many distinct signalling cascades. RhoA proteins have been identified as potential actors in the pathophysiology of the cardiovascular system. We used sucrose gradient fractionation and immunoblotting to determine caveolin-1 and RhoA presence in the kidney cortex of streptozotocin-induced T1 diabetes rats (4-week duration), and of diabetic rats treated with angiotensin receptor blocker losartan (4 weeks, 20 mg/kg/day) to retard renal hypertension. Positive RhoA/caveolin-1 co-immunoprecipitation result was detected in the caveolar fraction that corresponded to the light-scattering band obtained from diabetic rats, compared to negative co-immunoprecipitation result in the caveolar fraction obtained from control rats. The detection of RhoA protein in the caveolar fractions and the prospective RhoA/caveolin-1 association can be used to examine the role of these signalling reactions in the pathophysiology of microvascular complications in type 1 diabetes.

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Abbreviations: AT1R – angiotensin 1 receptor, BG – blood glucose, CAV-1 – caveolin-1, CAV-3 – caveolin-3, CONT – control animals, CONT-LOS – control animals medicated with losartan, DM-0 – group of streptozotocin/diabetic animals, DM-0-LOS – group of diabetic animals treated with losartan, DTT – dithiothreitol, ECL – enhanced chemiluminescence, EDTA – ethylenediaminetetraacetic acid; GAPs – GTPase-activating proteins, GDIs – guanine nucleotide dissociation inhibitors, GDP – guanosine diphosphate, GEFs – guanine exchange factors, GTP – guanosine triphosphate, HRP – horseradish peroxidase, MES – buffer with 2-(*N*-morpholino)ethanesulphonic acid, MM – molecular marker, PMSF – phenylmethanesulphonyl fluoride, RPM – revolutions per minute, SBP – systolic blood pressure, SDS – sodium dodecyl sulphate, SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis, T – Tween, T1D – type 1 diabetes, T2D – type 2 diabetes.

Introduction

RhoA protein belongs to the family of monomeric G proteins (small GTPases) on the basis of function and sequence identity, which in its active state binds GTP and in the inactive state GDP. Consequently, these GTPases ensure activation of the corresponding kinases. Specific guanine exchange factors (GEFs) reactivate GTPases by catalysing the replacement of GDP with a new GTP molecule. Other regulatory factors include GTPase-activating proteins (GAPs), which deactivate RhoA by enhancing its GTPase activity, and guanine nucleotide dissociation inhibitors (GDIs), which inhibit GAP's function, consequently suppressing RhoA's GTPase activity. Activated RhoA and Rac1 and Cdc42, other G monomeric GTPases, regulate actin cytoskeleton, thereby coordinating the cell shape, movement, secretion, endocytosis and the transport of cellular vesicles (Etienne-Manneville and Hall, 2002). Actin reorganization controls a wide spectrum of signal transduction mechanisms by interactions with effectors that are implicated in the regulation of gene expression and cell proliferation (Lufs et al., 2000). RhoA proteins are potential actors in the pathophysiology of the cardiovascular system as mediators of the mechanisms that operate in complications associated with type 1 and 2 diabetes (T1D, T2D) (Lauf and Liao, 2000). Evidences for the RhoA/Rho-kinase up-regulation in several hypertensive rats (Seko et al., 2003) and for the development of glomerulosclerosis in hypertensive rats have already been published (Kanda et al., 2003). Vasopressors such

as angiotensin II activate Rho-kinase by an essential mechanism involved in the changes of vascular tone (Takeda et al., 2001). In addition, high glucose activates RhoA/Rho-kinase and in response to high glucose, the matrix is up-regulated in mesangial cells and in diabetic glomeruli. Accumulation of glomerular matrix proteins is central to the pathogenesis of diabetic nephropathy (Peng et al., 2008).

Caveolae are plasma membrane invaginations in a variety of cell types (Anderson, 1998). Caveolae act as signalling platforms serving as concentrating points for numerous signalling molecules, as well as regulating flux through many distinct signalling cascades. Caveolin-1 (CAV-1) is the main structural protein component of the caveolae (Anderson, 1998; Frank et al., 2003). Localization of signalling molecules to caveolae has been shown to involve a direct interaction with the scaffolding domain of CAV-1 (Engelman et al., 1998). Protein-protein interactions of CAV-1 with these signalling molecules have major impact on their catalytic functions and intracellular targeting. As a major modulator of signal transduction, CAV-1 could play an important role in DM-induced alterations in intracellular signalling and enzymatic function, and consequently in the development of microvascular complications. Metabolic and humoral changes in T1D lead to haemodynamic, biochemical and structural changes in the kidney (Cooper, 1998). Factors characteristic of the diabetic metabolism, such as hyperglycaemia, abnormal glycosylation products, as well as altered lipids and fatty acids contribute to the changes in signal transduction pathways resulting in a wide spectrum of intracellular, biochemical changes culminating in altered gene expression within the affected cells. Alterations in the normal regulation of RhoA activity are likely to have major consequences in the pathophysiology of vascular complications and CAV-1 protein plays an important role in the modulation of this activity. The aims of this study were to investigate changes in the expression of RhoA protein and its co-distribution with CAV-1 in the experimental T1D and to examine the consequences of treatment with angiotensin receptor blocker, losartan.

Material and Methods

Diabetic rat model

Studies were conducted in adult male Wistar rats (Anlab, Prague, Czech Republic) with initial weights ~ 250 g. The rats were made diabetic by intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO), 65 mg/kg body weight. Three days later, induction of diabetes was confirmed by tail vein blood glucose (BG) measurements using a reflectance meter (One Touch II; Lifescan, Milpetas, CA). The animals were housed with a light-dark cycle of 12 h each, and with free access to food (standard chow) and water.

Study design

Diabetic rats were randomized to receive no treatment (DM-0, N = 6) and treatment with losartan (DM-0-LOS, 20 mg/kg/day in drinking water, N = 6) to retard renal hypertension. Age-matched non-diabetic Wistar rats served as two groups of controls, the first group of controls without any medication (CONT, N = 6) and the second group of controls with losartan (CONT-LOS, 20 mg/kg/day in drinking water, N = 6). Body weight, BG and systolic blood pressure (SBP, tail plethysmography) were measured at week 4 after induction of diabetes. Two to three days following these measurements, the rats were sacrificed with cervical dislocation and the kidneys were exposed via mid-abdominal incision, removed, divided into cortical and medullar portions, and snap frozen in liquid nitrogen for further analyses. All experiments were carried out with the approval of and in accordance with the regulations of the Institutional Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine.

Tissue fractionation on sucrose gradient

Renal cortical samples (100 mg) were homogenized in 1.5 ml 0.5 M Na_2CO_3 buffer (pH 11) containing 1 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 1 mM NaF, 10 nM okadaic acid, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenate (1 ml) was placed in a 12-ml ultracentrifuge tube and was adjusted to 45% sucrose by adding 1 ml of 90% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl). Sucrose gradient was prepared by adding 4 ml of 35% sucrose in MBS and 4 ml of 5% sucrose in MBS layered on top. Tubes were centrifuged at 260,400 *g* at 4 ºC for 24 h. Discontinuous sucrose density gradient centrifugation enables purification of the caveolae/lipid rafts because these microdomains have the propensity to "float" (Ishikawa et al., 2005). Light-scattering bands corresponding to the caveolar fractions (Lisanti et al., 1994; Song et al., 1996) were clearly visible after centrifugation. One millilitre samples corresponding to fractions 1–10 were collected from the top to the bottom of each tube (Lisanti et al., 1994; Song et al., 1996) and stored at –70 °C for further analyses. Protein concentration in each fraction was determined by the Lowry method (Lowry et al., 1951).

Western blot analysis

An equal volume of each fraction was mixed with loading buffer (0.5 M TRIS/HCl, 10% SDS, glycerol, 0.1% bromphenol blue) and boiled for 5 min, followed by western blot analysis as previously described (Komers et al., 2006). Briefly, denatured proteins were separated through an SDS-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were washed and then blocked overnight with TRIS-buffered saline, plus 0.05% Tween-20 (TBS-T) containing 5% non-fat dry milk. Following blocking, membranes were again washed, and incubated overnight with rabbit polyclonal

anti-CAV1 (N-20: sc-894, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1 : 800 in TBS-T and with rabbit polyclonal anti-RhoA (119: sc-179, Santa Cruz Biotechnology), diluted 1 : 400 in TBS-T (Santa Cruz Biotechnology). Immunodetection was accomplished by incubating membranes with a goat anti-rabbit-IgG secondary antibody conjugated with horseradish peroxidase (HRP, Pierce Biotechnology, Thermo Scientific, Rockford, IL) for 60 min (1 : 100,000) in TBS-T containing 5% non-fat dry milk. Visualization was performed with enhanced chemiluminescence (ECL) western-blotting kit (Supersignal West Dura, Pierce Biotechnology) according to the manufacturer's instructions. Photographic films (Eastman Kodak Co., Scientific Imaging Systems, New Haven, CT) were scanned using a flatbed scanner and images were analysed with NIH Image software.

Co-immunoprecipitation

The antigen-antibody complex (immune complex) was formed by incubating 200 µg protein from each fraction overnight with rabbit polyclonal anti-CAV1 (1 : 50) at 4 °C in rotating tubes. Immune complexes were captured in immobilized Protein A/G agarose gel (Santa Cruz Biotechnology) supported by incubation for 2 h at 4 °C. The unbound proteins (non-immune complex sample components) from the precipitated complex were removed by washing. Loading buffer was added to the pellets and mixtures after boiling were analysed by SDS-PAGE. Rabbit polyclonal anti-RhoA (Santa Cruz Biotechnology) was used for the detection. Benchmark pre-stained protein ladder (Invitrogen, Carlsbad, CA) was used as a molecular marker (MM) for both western blots and co-immunoprecipitation methods. Photographic films (Eastman Kodak Co., Scientific Imaging Systems) were scanned using a flatbed scanner and images were analysed with NIH Image software. The intensities of association/interaction between samples with and without antibody from all rats were statistically analysed by Mann-Whitney test. CAV-1, RhoA and RhoA/CAV-1 determination in all fractions obtained from each rat were performed at least in triplicate.

Results

Diabetic rats without losartan treatment demonstrated higher systolic blood pressure, reduced weight gain and renal hypertrophy. Systolic blood pressure and renal hypertrophy was markedly reduced by treatment with losartan. General physical parameters and laboratory results of the CONT and CONT+LOS group did not differ in any aspect of the experiment.

Renal cortical samples were fractioned on a discontinuous sucrose gradient and the obtained fractions (1 ml) were analysed for RhoA presence and immunoreactivity. Caveolar membrane fractions (fraction 5 and fraction 6) corresponded to light-scattering bands originating after ultracentrifugation in discontinuous sucrose gradient. As can be observed in Fig. 1, in the samples obtained from CONT (CONT+LOS) animals, RhoA

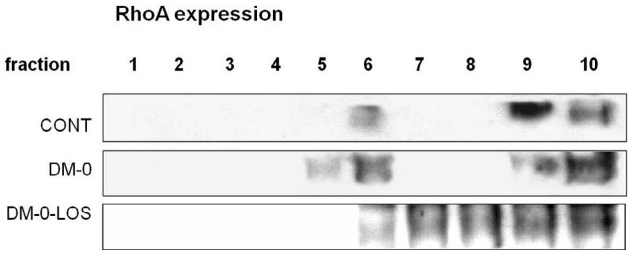

Fig. 1. Picture shows representative images of RhoA expression analysed by western blotting in subsequent (1–10) fractions in control rats, in diabetic rats without pharmacological interventions (DM-0) and in diabetic rats treated with angiotensin receptor blocker losartan (DM-0-LOS).

protein was enriched in caveolar fraction 6 and in fractions 9 and 10, which sedimented in the higher sucrose density. In the samples obtained from DM-0 diabetic rats, RhoA protein was enriched in caveolar fractions 5 and 6 and in fractions 9 and 10. In the samples obtained from DM-0-LOS diabetic rats, RhoA protein was enriched in fractions 6, 7, 8, 9 and 10 (Fig. 1). RhoA protein was observed in the caveolar membrane fractions obtained from DM-0 rats in higher quantity compared to CONT animals (Fig. 2); however, the intensity of expression was not significant. It is also observable that RhoA protein was more abundant in the caveolar fractions obtained from DM-0 rats compared to the samples obtained from DM-0-LOS; the intensity of expression was significant (P = 0.013) (Fig. 2). RhoA protein was detected in DM-0-LOS rats in large amounts in the fractions that sedimented in the higher sucrose density. The finding was apparent despite the total protein content tendency; total protein content in the caveolar fractions obtained from the DM-0 group was very low (0.4–0.8 µg/µl); total protein content in the caveolar fractions obtained from the DM-0-LOS was higher (1.2–1.8 µg/µl).

Co-immunoprecipitation analysis demonstrated RhoA/CAV-1 protein association in different conditions.

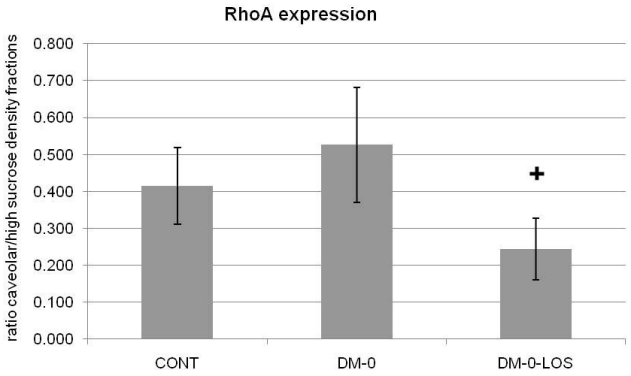

Fig. 2. Graph shows comparable ratio of RhoA expression in the caveolar membrane fractions obtained from control rats, diabetic rats without pharmacological interventions (DM-0), and from diabetic rats treated with angiotensin receptor blocker losartan (DM-0-LOS). Results are performed in means and standard deviations. DM-0 vs. DM-0-LOS (P = 0.013)

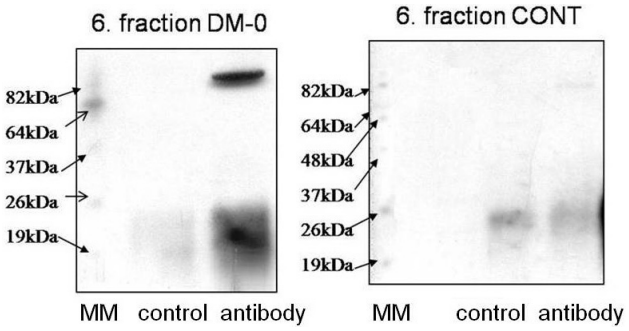

Fig. 3. Picture shows representative images of RhoA/CAV-1 co-immunoprecipitation analysed in the caveolar fraction 6 obtained from control rats and from diabetic rats without pharmacological interventions (DM-0). Analyses demonstrate evident difference between samples with and without anti-CAV1 antibody in the caveolar fraction 6 obtained from DM-0 rats, but an absence of difference in the caveolar fraction 6 obtained from control rats (CONT). Benchmark pre-stained protein ladder was used as a molecular marker (MM).

In the caveolar fraction 6, which corresponded to the light-scattering band obtained from DM-0 rats, the reaction was more pronounced compared with the reaction in the caveolar fraction 6 obtained from CONT animals (Fig. 3); the difference was significant comparing intensity of expression between samples with and without antibody (Fig. 4). In the caveolar fraction 5 obtained from the DM-0 group, there was weak positive reaction. In other fractions obtained from CONT animals, DM-0 and DM-0-LOS groups, no other significance or significant differences provided by the co-immunnoprecipitation method have been proved. Images of these experiments are not presented.

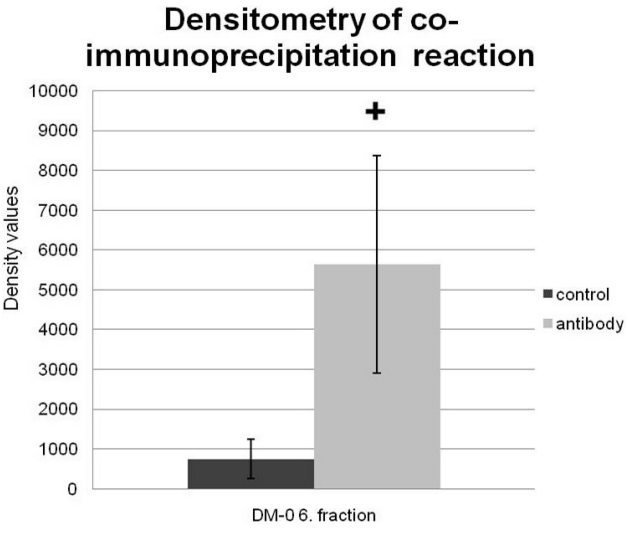

Fig. 4. Graph shows densitometry values of RhoA/CAV-1 co-immunoprecipitation reaction in the fraction 6 obtained from the DM-0 group. Sample without vs. with antibody (P = 0.0358)

Discussion

Using discontinuous sucrose fractionation we have previously identified caveolar fractions in cortical samples that corresponded to light-scattering bands (Lisanti et al., 1994; Song et al., 1996, Demova and Komers, 2009), and CAV-1 protein expression under diabetic conditions without and with losartan treatment (Demova and Komers, 2009). The sucrose fractionation method addresses the question of co-localization of other proteins that undergo caveolar translocation in response to a variety of physiological and pathophysiological stimuli. A growing and varied list of signalling molecules have been identified as residing in the caveolae. Interactions with caveolin are believed to sequester these proteins within caveolae and modulate or suppress their catalytic activities (Razani and Lisanti, 2001; Razani et al., 2002). Therefore, we used the western blotting method with anti-RhoA antibody to demonstrate RhoA expression in the discontinuous sucrose fractions of all groups of rats. The RhoA protein was enriched in both caveolar and higher sucrose density fractions obtained from both CONT (CONT+LOS) and DM-0 rats; however, the expression of RhoA was higher in the caveolar membrane fractions of diabetic rats. Kawamura et al. (2003) detected a significant portion of RhoA protein in both caveolar and higher sucrose density fractions in both stretched and unstretched cardiomyocytes with similar intensity of expression (Kawamura et al., 2003). Gingras et al. (1998) detected a significant portion of RhoA protein in both caveolar and higher sucrose density fractions in endothelial (ECV304 line of T24 bladder carcinoma) cells using detergent-free floatation analysis (Gingras et al., 1998).

Recent projects suggest that RhoA is observed predominantly in the cytosolic fraction in control conditions, and it is translocated to the particular membrane fraction in response to activation (Bokoch et al., 1994; Aoki et al., 1998; Kataoka et al., 2002; Lecian et al., 2006). We have proved increased RhoA expression in the caveolar membrane fractions obtained from diabetic rats without treatment; however, the difference was not significant. Also, we have previously demonstrated a significant increase of CAV-1 protein levels in the caveolar fractions obtained from the DM-0 group (Demova and Komers, 2009). The same increase of CAV-1 and CAV-3 protein was reported by Pascariu et al. (2004) in endothelial cells of diabetic rats and by Kawamura et al. (2003) in stretched cardiomyocytes. To support the hypothesis that during early stage of diabetic nephropathy there is an increased association of CAV-1 and RhoA protein in cortical cells, we employed the co-immunoprecipitation method with anti-CAV-1 antibody and after that western blotting with immune complexes and anti-RhoA antibody. In the caveolar fraction 6 obtained from the DM-0 group there was strong positive reaction in comparison to the caveolar fraction 6 obtained from the CONT animals. The presence of RhoA protein was demonstrated in the caveolar membrane fractions in

both CONT and diabetic rats (Gingras et al., 1998; Kawamura et al., 2003); however, only in diabetic rats very strong association between RhoA and CAV-1 proteins was proved. Peng et al. (2007) used the co-immunoprecipitation method with mesangial cells stimulated by mechanical strain with similar results; no significant association between RhoA and CAV-1 was observed at baseline. However, stretch led to a significantly increased association between RhoA and CAV-1, with maximal association occurring at the time of maximal RhoA activation (Peng et al., 2007).

It was strongly suggested that translocation of RhoA to caveolae and its association with caveolin-1 may facilitate the subsequent activation of Rho kinase (Michel et al., 1997; Shaul and Anderson, 1998; Teixeira et al., 1999; Dubroca et al., 2007). We were not able to provide another method to confirm this statement in our diabetic rats; however, in our study the co-immunoprecipitation method was clearly negative in the DM-0-LOS group. In the sample obtained from DM-0-LOS rats, RhoA protein was present in a larger degree in the fractions that sedimented in the higher sucrose density; therefore, it was represented by not activated cytosolic Rho proteins (Gingras et al., 1998). Both patients and diabetic rats suffer from renal hypertension during early stage of diabetic nephropathy. Losartan is indicated for the treatment of hypertension and for the treatment of advanced renal insufficiency (Osawa et al., 2006). A change in the vascular tone caused by angiotensin II and its reaction with the receptor induced an intracellular cascade of protein reactions, which involved activation of the Rho-kinase, target of the small RhoA GTPase (Calo and Pessina, 2007). It is therefore conceivable that interaction of RhoA with caveolin-1 leads selectively to the activation of a Rho kinase-dependent force development (Kawamura et al., 2003); and blockade of angiotensin I receptor (AT1R) by losartan reduces RhoA expression in the caveolar membrane fractions and RhoA association with CAV-1 protein.

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