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INCREASED ABILITY OF ERYTHROCYTES TO AGGREGATE IN SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract

The development of hypertension is accompanied by changes in the rheological properties of blood, particularly by increased red blood cell (RBC) aggregation leading to further pathological complications. However, it is not clear whether these changes in aggregation are caused only by increased concentrations of plasma adhesion proteins or if alterations in RBC membranes are also involved. The aim of the present study was to determine if RBC aggregability is altered during hypertension and if these changes correlate with alterations in RBC membrane protein concentrations. Aggregability changes were evaluated by comparing fibrinogen (Fb)-induced aggregation of RBCs from spontaneously hypertensive rats (SHR) with RBCs from age matched normotensive Wistar Kyoto (WKY) rats. ANOVA showed a significant increase in dose-dependent Fb-induced aggregation of RBCs in the SHR group. Analysis of Coomassie-stained gels of RBC membrane proteins using SDS-PAGE showed a significant increase in the amount of a protein at 110 kD in the SHR group. These results show that increased RBC aggregability is accompanied by alterations in RBC membrane protein composition during hypertension development.

Keywords

Erythrocytes; Fibrinogen; Hypertension; Membrane proteins

INTRODUCTION

Blood viscosity has been found to be one of the strongest predictors for cardiovascular events. [1] An increase in blood viscosity is the result of changes in a number of parameters including increases in hematocrit, plasma viscosity and RBC aggregation. In vitro alterations in these blood rheological variables during hypertension have been extensively reported.[1-7] There were several reports suggesting involvement of blood rheology alterations in the development of hypertension.[4,7-9] Arterial pressure is dependent on cardiac output and total peripheral vascular resistance (TPVR) with the latter being determined by the caliber of resistance vessels and the intrinsic viscosity of blood. It has been shown that cardiac output is generally normal in established hypertension.[10] Thus, in hypertension the elevation of blood viscosity is accompanied by a normal vascular hindrance[4] without compensatory vasodilation.[4,11, 12] Therefore, elevated TPVR is the main cause of increased arterial blood pressure.[9]

Although there were several convincing studies in the past illustrating that an increase in RBC aggregation causes a decrease in local blood flow in microvessels,[13-16] this concept was completely ignored until the last decade. Several laboratories have reported that an increase in RBC aggregation caused an elevation in total vascular resistance to blood flow.[17-20] Cabel et al. have shown that RBC aggregation-induced blood flow viscosity changes are responsible for alteration in venous resistance to flow.[21] In addition, it was shown that RBC hyperaggregability-induced blood flow resistance was increased in arteriolar[20] and capillary parts[20,22] of the microcirculation.

Alterations of major determinants of the blood's rheological properties observed in hypertensive disease include increased viscosity of blood[23,24] and plasma,[23-25] increased hematocrit[24,25] and increased levels of high molecular weight plasma proteins including fibronectin,[25] immunoglobulin-A[26] and particularly fibrinogen (Fb),[3] which are the major determinants of plasma viscosity. Furthermore, it has been shown that RBC aggregation, which has the most significant effect on blood rheological properties, is increased during hypertension.[23-27] Among the plasma adhesion proteins having an effect on RBC aggregation, Fb has been identified as having the most pronounced effect.[3,23,26-28]

While the relationship between plasma proteins and RBC aggregation is established, the mechanism of action is still unclear. According to the prevailing hypothesis, RBC aggregation is thought to be caused by nonspecific protein binding to RBCs either by molecular bridging [28-30] or by protein depletion[28,31] mechanisms. In the former nonspecific adhesion theory, the aggregation depends on the molecular mass of the adhesion proteins and on the surface adsorption,[29] while in the latter case, the aggregation is independent of both the molecular mass and the surface adsorption.[32] However, the discovery of specific receptors for ceruloplasmin on RBCs[33] and our finding of Fb specific binding to RBC membrane[34] suggest the possible co-existence of receptor mediated mechanisms. In the current study we present data showing that RBC membrane protein expression is altered during hypertension. This finding raises the possibility of yet another RBC membrane determined mechanism for enhanced RBC aggregation seen during hypertension.

METHODS

Animals and Blood Collection

Male spontaneously hypertensive rats (SHR; $n = 10$) of the Okamoto and Aoki strains and normotensive Wistar-Kyoto (WKY; $n = 9$) rats were used at 12 weeks of age. After anesthesia (sodium pentobarbital, 50 mg/kg; i.p.), a tracheal cannula was inserted to maintain a patent airway, and a carotid artery cannula was used to continuously (for 1 h) monitor mean arterial blood pressure and diastolic pressure with a Micro-Med blood pressure analyzer (Louisville, KY) in SHR and WKY rats. Then about 7 mL of blood were withdrawn by venipuncture of the *vena cava* using syringes containing sodium citrate anticoagulant (10.9 mmol/L) with a ratio of 1 part of anticoagulant to 9 parts of blood. The blood was centrifuged at $2000 \times g$ for 10 min at room temperature to obtain blood plasma for Fb concentration measurements and for RBC aggregation assessment in homologous plasma. A blood hematocrit was determined using a microhematocrit centrifuge. The RBCs were washed 4 times in phosphate buffered saline (PBS) (42.6 mmol/L Na_2HPO_4 , 7.4 mmol/L NaH_2PO_4 , 90 mmol/L NaCl, 5 mmol/L KCL, 5 mmol/L glucose, pH = 7.4; 285 mosmol) by centrifugation at $3000 \times g$ for 5 min each time. Then the cells were used either for preparation of RBC ghosts, for assessment of RBC aggregation in homologous plasma or for evaluation of RBC aggregability.

RBC Aggregation and RBC Aggregability Assessment

To evaluate RBC aggregation during hypertension we modified the method, which was described previously.[25] The washed cells were suspended in homologous plasma with a volume ratio of 1 part of erythrocytes to 200 parts of plasma. An assessment of plasma-induced RBC aggregation was done under static conditions by direct visualization of the process.[25]

For RBC aggregability evaluation, human plasma Fb (FIB-3; Enzyme research Laboratories, South Bend, IN) was diluted in a PBS solution at concentrations of 2, 4, 6, 8, 12 and 16 mg/mL. Then the thoroughly washed RBCs were suspended in these PBS-Fb solutions at a 1 : 200 ratio. As a control, RBC aggregation in PBS alone was evaluated. An image analysis program (Matrox Inspector-3, Matrox Imaging, Dorval, Canada) was used to determine the degree of RBC aggregation in the samples. RBC aggregation was presented as the Erythrocyte Aggregation Index (EAI), which is defined as a ratio of the total area of aggregates to the total area of all RBCs expressed as a percent.[25] The alterations in RBC aggregability were assessed by differences between Fb-induced EAI of RBCs from SHR and WKY groups at each concentration of Fb.

Preparation of RBC Membranes

The washed RBCs were mixed with 9 volumes of ice-cold lysis buffer (5 mmol/L sodium phosphate) and stirred for 15 min at 0°C. Subsequently the unsealed RBC ghosts were pelleted by centrifugation at 37,000 xg for 10 min at 0°C. After the centrifugation, the ghosts were washed with ice-cold lysis buffer until residual hemoglobin was not visible. The RBC ghosts were suspended in 0.5 volume of 50 mmol/L PBS and were kept frozen at -80°C until use.

Analysis of Coomassie Stained SDS PAGE Gels

SDS-PAGE analysis of the membrane proteins of erythrocytes from SHR ($n = 5$) and WKY rats ($n = 4$) was performed according to the method described previously.[35] Coomassie blue (Bio-Rad, Hercules, CA) stained gels were analyzed for protein concentration of the bands with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD). The protein expression intensity was assessed by Integrated Optical Density (IOD), i.e. the area of the band in the lane profile. To account for possible differences in total protein load, the results of the measurements are presented as a ratio of IOD of each band under the study to the IOD of β spectrin (band 2).

Protein Concentration Measurements in Samples

Fb concentration in plasma samples was measured as described previously.[25] All other protein measurements utilized the Pierce (Rockford, IL) BCA kit with bovine serum albumin as a standard.

Statistical Analysis

Values given in the text and figures are the means $\pm$ SEM. A two-way ANOVA was used to assess the effects of hypertension and increasing concentrations of Fb on RBC aggregability. Differences between other experimental data were evaluated by the nonparametric Mann-Whitney Rank Sum Test. Statistical significance was assumed at a value of $P < 0.05$.

RESULTS

Comparisons of SHR with their age matched control WKY rats demonstrated significant increases in all the measured variables: mean arterial pressure [SHR (165 ± 4 mm Hg) vs. WKY (81 ± 4 mm Hg)], diastolic pressure [SHR (145 ± 5 mm Hg) vs. WKY (65 ± 4 mm Hg)], hematocrit [SHR ($49 \pm 2\%$) vs. WKY ($41 \pm 2\%$)], plasma-induced EAI [SHR ($53 \pm 5\%$) vs.

WKY ($13 \pm 1\%$), and plasma Fb concentration [SHR (4.0 ± 0.3 mg/mL) vs. WKY (3.0 ± 0.2 mg/mL)].

As expected, Fb-induced RBC aggregation in the absence of plasma was progressively increased with increases in Fb concentration in all RBC-PBS samples from both groups of rats (Fig. 1). However, two-way ANOVA shows a significant increase in Fb-induced EAI values for hypertensive rats compared to normotensive controls starting from a Fb concentration of 4 mg/mL (Fig. 1). The significant differences between these values at each concentration of Fb studied demonstrate an increase in erythrocyte aggregability in SHRs (Fig. 1). These results are in agreement with similar findings in our previous study from hypertensive patients[36] and suggest that this phenomenon is not limited to hypertension in rats.

Protein expression in RBC membranes was analysed by SDS PAGE (Fig. 2). Out of 35 visible bands, three (bands marked as a, b and c on Fig. 2) were noticeably different in the hypertensive group. The ratio of IOD of a band with a molecular weight of about 110 kD (band c) to IOD of the reference protein-band 2 (see Fig. 2) was significantly higher in RBC membranes from the SHR group (0.65 ± 0.07 ; $P < 0.05$) compared to the WKY group (0.39 ± 0.09) (Fig. 3). The ratio of IOD of the bands with molecular weights of 134 (band a) and 121 kD (band b) to IOD of band 2 tended to be increased in the SHR group (0.70 ± 0.08 ; $P = 0.06$ and 0.72 ± 0.17 ; $P = 0.22$ respectively) compared to the similar ratios bands of the same molecular weight in the WKY group (0.45 ± 0.07 and 0.42 ± 0.14 respectively; Fig. 3).

DISCUSSION

The results of the present study are in agreement with the data reported elsewhere for hypertensive humans and animals,[1-10,23-27] and demonstrate that plasma Fb concentration was increased in SHRs compared to the normal level of about 3 mg/mL in WKY rats. This significantly higher concentration of Fb (4 mg/mL) may be a factor in the increased RBC aggregation in homologous plasma in hypertensive rats (Results; 25). RBC aggregability, which is determined solely by the properties of RBCs themselves, and which was evaluated by comparison of EAI between the groups of Fb-induced aggregation of RBCs at each concentration of Fb, was also increased during hypertension (Fig. 1). Interestingly, the greater Fb-induced RBC aggregation in SHRs compared to the WKY group is exhibited starting at a Fb concentration of 4 mg/mL (Fig. 1), which is the typical plasma Fb concentration for SHRs (Results; 25). Since this increase in RBC aggregability is at a given Fb concentration (e.g. 4 mg/mL), our results suggest that the mechanism of Fb/RBC interaction is altered due to changes in RBC properties during hypertension rather than the increase in Fb observed in SHRs.

It is well known that the viscoelastic properties of RBC membranes, RBC deformability, interactions between macromolecules and RBCs as well as hematocrit each have an effect on RBC aggregation.[30,37] To avoid the effect of differences in hematocrit in the samples, we used standardized hematocrits of washed RBCs in PBS (1 : 200). Therefore, the results of the present study suggest that increased hematocrit and plasma concentration of Fb are not involved in increased RBC aggregability during hypertension (i.e. data 4 mg/mL Fb, Fig. 1). In addition, Chabanel et al. have shown that RBC elasticity (deformability) of 9–23 weeks old SHRs is similar to that of age matched normotensive rats.[5] Combined, our results and this previous report[5] imply that there are other factors contributing to increased RBC aggregability and led us to test for the existence of differences in RBC membrane protein contents (which may also contribute to the RBC surface charge) during hypertension. Indeed, we found that an RBC 110 kD membrane protein is over expressed in SHRs (Figs. 2 and 3). Others have shown that a 110 kD membrane protein is a glycoprotein[38] and may be an integrin type protein.[39] These results suggest the possible co-existence of an additional, RBC membrane-based mechanism that may have an effect on RBC aggregation during hypertension.

In conclusion, the results of the present study demonstrate that increased RBC aggregability in hypertensive rats may be the result of changes in RBC membrane protein expression. These changes, in addition to increased plasma Fb concentration may result in an increase in RBC aggregation, and therefore, in alterations of blood rheological properties that may contribute to increased peripheral vascular resistance and lead to further complications during hypertension.

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Figure 1.

Comparison of fibrinogen dose-response changes in RBC aggregation for SHR and WKY rats. ANOVA demonstrates a significant difference in the hypertensive group compared to the control response (see text for the details).

Figure 2.

SDS PAGE analysis of RBC membrane proteins from SHR and WKY rats. Samples (30 μ g) of each membrane preparation were submitted to SDS-PAGE. The gels were then stained with Coomassie blue, destained by free diffusion in 7% acetic acid, and dried under vacuum (top). The portion of the gel from the origin to approximately 70 kD is shown. Lanes 1, 2, 5, 6, and 7 are from SHR and lanes 3, 4, 8, and 9 are from WKY rats. Result of a typical gel scan using the Gel-Pro Analyzer software (bottom). RBC membrane proteins with molecular weights of 134, 121 and 110 kD are identified by arrows and marked by letters a, b and c, respectively. These proteins are located between the well characterized RBC membrane proteins band 2 (β spectrin) and band 3 (shown by arrows).

Figure 3.

Comparison of expression levels of 134, 121 and 110 kD RBC membrane proteins from SHR and WKY rats. The data are presented as ratios of integrated optical density (IOD) of each band to the IOD of band 2 from the same sample. Optical Density was determined using the Gel-Pro Analyzer software as shown in Fig. 2 (bottom). * $P < 0.05$ for comparison between the groups.