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REGULAR ARTICLE

Alteration in protein kinase B (AKT) activity in platelets from patients with systemic sclerosis

Thomas M. Chiang [a,b,c,*], **Arnold E. Postlethwaite** [a,b]

[a] *Veterans Affairs Medical Center, 1030 Jefferson Avenue, Memphis, TN 38104, USA*
[b] *Department of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA*
[c] *Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, TN 38163, USA*

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Abstract

We have previously reported that there is an increase in phosphotidylinositide-3 kinase activity secondary to increase in nitrotyrosylation in platelets from patients with SSc. Protein kinase B (Akt) is recruited to the plasma membrane by PI 3-K metabolites. Both enzymes play critical roles in signal transduction and activation of platelets. In the present investigation, we have studied the effect of postranslational modification of the activity of Akt. We have examined eight patients and eight controls and obtained results showing that enzymatic activity of Akt is increased in lysates of platelets from patients with SSc compared to normal volunteer controls. We have obtained results showing that there is no correlation of nitrotyrosylation of Akt on its enzymatic activity although Western blots show the enzyme has increased nitrotyrosylation. These results suggest that post-translational modification by nitrotyrosylation does not control Akt in SSc platelets. We concluded that the enhanced activity of PI 3-K and Akt in platelets from patients with SSc is mediated by different mechanisms.
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Abbreviations: SSc, systemic sclerosis; PI 3-K, phosphatidylinositol 3-kinase; Akt, protein kinase B; NO, nitric oxide; PRP, platelet-rich plasma; TBST, 20 mM Tris–HCl/500 mM NaCl/0.05%; Tween 20, pH 7.4; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; TMB, tetramethylbenzidine; and SIN-1, 3-morpholinosydnonimine-1; eNOS, endothelial form of nitric oxide synthase; cGMP, guanosine cyclic 3′:5′-monophosphate; VWF, von Willebrand factor; and GP IIb/IIIa, glycoprotein IIb/IIIa.

* Corresponding author. Research Service (151), Veterans Affairs Medical Center, 1030 Jefferson Avenue, Memphis, TN 38104, USA. Tel.: +1 901 523 8990x7608; fax: +1 901 577 7273.
E-mail address: tchiang@utmem.edu (T.M. Chiang).

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Introduction

Microvascular injury is one of the major pathogenetic processes involved in systemic sclerosis (SSc)[#]. Interaction of the platelets with collagen in the exposed subendothelial stroma leading to platelet activation and aggregation results in on-going microvascular injury in SSc. In addition, this activation–aggregation process results in the release of mediators, several of which are potent fibrogenic agents, which are believed to be important in driving fibrogenesis in SSc. The mechanism of the activation has not been well defined in SSc platelets. Although, we have reported that there is an increase in both platelet non-integrin receptor and phosphatidylinositol 3-kinase (PI 3-K) activity of SScs platelets [1,2].

Many factors control the activation of platelets. One of the factors is the metabolism of phosphoinositides, which has been known to play a critical role in the platelet signal transduction. PI 3-K is an enzyme which catalyzes the phosphorylation of phosphatidylinositol 4, 5 to phosphatidylinositol 3, 4, 5-trisphosphate. Phosphoinositides have been shown to control the reorganization of actin cytoskeleton [3,4] and are required for store-mediated Ca^{2+} entry in platelets [5]. It has also been reported that lipid products of PI 3-K and PI 4-K are both required for ADP-dependent platelet spreading [6].

Rheological disturbances have an important role in promoting arterial thrombosis by enhancing the adhesive and signaling function of platelet. Jackson et al. [7] have reported that PI 3-K has a defined role in regulating the formation and stability of the adhesion process, which is necessary for activation of platelets. The closely linked enzyme of PI 3-K is the protein kinase B (Akt), which will collaborate the signal transduction of platelets.

Peroxynitrite anion [$ONOO^-$] is a potent oxidant generated from the interaction of nitric oxide [NO] and superoxide [O·2] [8–10]. Under the physiological conditions, NO is the only known biological molecule that can out-compete endogenous superoxide dismutase for available O2 [11] and formation of $ONOO^-$ can account for both O_2^- and NO-dependent toxicities [10]. Formation of $ONOO^-$ has been reported in cells [11–14]. Peroxynitrite reacts with a diverse array of other biological target molecules, including cysteine, tyrosine, methionine, and tryptophane residues of proteins [15].

We have reported that the activity of platelet PI 3-K is controlled by nitrotyrosylation and the enzyme activity is increased in SSc patients [2]. The other adjacent component of the platelet signal pathway is the protein kinase B (Akt) [16]. Is the Akt also posttranslationally altered in SSc patient's platelets? In the present investigation, we observed that nitrotyrosylation is not a controlling mechanism for the activity of Akt in platelet lysates of systemic sclerosis (SSc) patients compared to that of normal controls. The activity of Akt is regulated by phosphorylation. These results suggest that there are different mechanisms to control PI 3-K and Akt in SSc patients' platelets.

Materials and methods

Materials

All chemicals were purchased from Fisher Scientific (St Louis, MO), Sigma Chemical Co. (Denver, PA), and BioRad (Hercules, CA). Anti-phosphoserine/threonine was purchased from Upstate Biotechnology, (Lake Placid, NY). Anti-Akt was purchased from Upstate Cell Signalling (Charlottesville, VA). Akt ELISA assay kit was purchased from Stressgen (Victoria BC, Canada). Enhanced chemiluminescence (ECL) solutions were from Amersham Biosciences Corp. (Piscataway, NJ).

Patients and controls

The Institutional Review Boards of the University of Tennessee, Memphis and VA Medical Center approved the protocol for this study. After informed consent was given to each participant, blood samples from SSc patients and controls were collected; platelets were isolated from blood, and kept at −80 °C freezer until use. Disease period, sex, and age of these patients have been published in an earlier manuscript [1].

Preparation of platelet-rich plasma (PRP)

Blood (9 parts) from SSc patients and healthy volunteers was collected in polypropylene tubes containing 3.8% sodium citrate (1 part). PRP was prepared by centrifuging the citrated blood at room temperature for 10 min at 226 × g [17].

Immunoblot

Platelets were harvested from PRP and washed with PBS by centrifugation and were resuspended in 20 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors (25 μM aprotinin, 25 μM leupetin, and 1 μM PMSF). The suspension was sonicated for 20 s at 4 °C, and the protein concentration was determined. An aliquot of platelet lysates (100 μg) plus an equal volume of SDS-PAGE sample buffer were mixed, boiled for 3 min and analyzed by 10% SDS-PAGE [18]. Protein bands were electrophoretically transferred onto a nitrocellulose membrane, followed by treatment with 3% dried milk in TBST (20 mM Tris-500 mM NaCl-0.05% Tween 20, pH 7.4) to eliminate non-specific binding. The nitrocellulose sheet was then washed 3 times with TBS and incubated with anti-nitrotyrosine antibody (1/1000) overnight at 4 °C. After washing 3 times with TBST, the nitrocellulose sheet was incubated with a second antibody (peroxidase conjugated goat anti-mouse IgG, 1/20000) for 2 h at room temperature. After three washings, the nitrocellulose sheet was developed with an enhanced chemiluminescence (ECL) solutions according to the manufacturer's description as we have used routinely in our laboratory [2].

Immunoprecipitation of Akt

We have used immunoprecipitation to partially purify the Akt from platelet lysates to ensure the activity we obtained is Akt. An aliquot of platelet lysates (50 μg) was mixed with anti-Akt antibody (1/50) and incubated at 4 °C overnight. A second antibody (1/50), goat anti-rabbit IgG was added to the mixture