Prostaglandins & other Lipid Mediators 116–117 (2015) 131–135

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

Original Research Article

Prostacyclin receptor expression on platelets of humans with type 2 diabetes is inversely correlated with hemoglobin A1c levels

Stephanie M. Knebel, Randy S. Sprague, Alan H. Stephenson*

Department of Pharmacological and Physiological Science, Saint Louis University, St. Louis, MO 63104, United States

ARTICLE INFO

Article history:
Available online 21 January 2015

Keywords:
Prostacyclin receptor
Platelet
cAMP
Type 2 diabetes
Radioligand binding
Western blot

ABSTRACT

Inappropriate platelet aggregation can result in thrombosis and tissue ischemia. When compared to healthy human platelets, those of humans with type 2 diabetes (DM2) exhibit increased aggregation when stimulated. Activation of the platelet prostacyclin receptor (IPR) results in cAMP accumulation and inhibition of platelet aggregation. We hypothesized that DM2 platelets express decreased IPR when compared to platelets of healthy humans, resulting in decreased IPR agonist-induced cAMP accumulation. We measured IPR expression with radioligand binding of [^{3}H]-iloprost, a stable prostacyclin analog, and with Western blotting of the IPR protein. Iloprost-stimulated platelet cAMP levels were used to identify the functional response to IPR activation. IPR binding, expression of the IPR protein and the levels of cAMP in platelets incubated with iloprost were significantly decreased in DM2 platelets when compared to platelets of healthy humans. IPR expression decreased in platelets as glycemic control of the subjects worsened, as indicated by increased hemoglobin A1c levels. Taken together, these findings suggest that reduced IPR expression in DM2 platelets may contribute to platelet hyperactivity in humans with type 2 diabetes.

1. Introduction

Atherothrombotic disease affecting the coronary vessels, cerebral circulation and lower limb peripheral arteries is accelerated in subjects with type 2 diabetes [1–4]. It has been proposed that heightened platelet activation contributes to these events [1,2,5]. The evidence supporting this proposal is derived from studies demonstrating that platelets obtained from humans with type 2 diabetes aggregate more readily to stimulation with prothrombotic agonists such as collagen, ADP and platelet activating factor [1,6–8]. Moreover, platelets of humans with type 2 diabetes are less sensitive to the anti-aggregatory effects of prostacyclin [8–10] and nitric oxide [11,12]. This defect is exacerbated by the fact that the vascular endothelium of humans with type 2 diabetes releases decreased amounts of these important mediators [11,13,14]. Although many studies have focused on mechanisms that mediate the increased platelet activation associated with diabetes [15], fewer studies have identified possible mechanisms that mediate the decreased sensitivity to prostacyclin or nitric oxide. In the present study, we hypothesized that one mechanism responsible for decreased sensitivity to prostacyclin in platelets of humans with type 2 diabetes may result from decreased expression of the prostacyclin receptor (IPR). We investigated this hypothesis by comparing IPR expression by both radioligand binding and IPR protein measurements from Western blots in platelets of healthy humans with that of humans with type 2 diabetes. As a functional consequence of altered IPR expression, we also examined the ability of these platelets to increase cyclic AMP levels in response to iloprost, a stable IPR agonist.

2. Methods

2.1. Isolation of platelets from whole blood

Human blood was obtained by venipuncture and collected into a syringe containing heparin (500 U/30 ml of blood). Blood from humans with type 2 diabetes was obtained from patients at the Endocrinology Clinic at Saint Louis University Hospital. Hemoglobin A1c (HbA1c) levels were determined using 10 μl of whole blood with an A1cNow® kit (Bayer Healthcare). Blood was centrifuged at 300 × *g* for 10 min at 4 °C. The platelet rich plasma

* Corresponding author at: Saint Louis University, Department of Pharmacological & Physiological Science, School of Medicine, 1402 South Grand Blvd, St. Louis, MO 63104, United States. Tel.: +1 314 977 6400; fax: +1 314 977 6410.
E-mail addresses: stephmknebel@gmail.com (S.M. Knebel), spraguer@slu.edu (R.S. Sprague), stephens@slu.edu (A.H. Stephenson).

http://dx.doi.org/10.1016/j.prostaglandins.2014.12.002
1098-8823/

was removed and added to a tube containing an additional 500 U heparin in acid-citrate-dextrose (ACD) solution to prevent platelet aggregation [16] and centrifuged for 20 min at 1000 × *g* at 4 °C. After centrifugation, the supernatant was aspirated and discarded. The platelet pellet was resuspended in ACD solution, and centrifuged for an additional 20 min at 1000 × *g* at 4 °C. The supernatant was discarded and the pellet was resuspended in assay buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, final pH 7.4 at 4 °C) for radioligand binding assays and cAMP assays, or the pellet was resuspended in Western blot extraction buffer (25 mM HEPES, 300 mM NaCl, 10 mM EDTA, tetrasodium, 1.5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, final pH of 7.4 at 4 °C). One tablet of Complete® Protease Inhibitor Cocktail (Roche) was added per 10 ml of Western blot extraction buffer. Blood from all subjects was obtained with informed consent and the protocol for its removal was approved by the Institutional Review Board of Saint Louis University.

2.2. Platelet IPR binding experiments

Platelets, lysed by sonication at 4 °C were suspended in assay buffer (400 μg of protein/100 μl of lysate) and aliquotted into siliconized glass tubes. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay (Pierce). [^{3}H]-iloprost (1–100 nM) was added to reaction tubes, followed by unlabelled iloprost or vehicle (saline). Platelets were incubated for 20 min at room temperature, followed by 15 min at 4 °C. Following incubation, the mixture was rapidly vacuum-filtered (Millipore) through a glass microfiber filter (GF/C, Whatman), pre-soaked with buffer. The filters were rapidly washed twice with 4 °C buffer (1 ml). Washed filters were placed inside scintillation vials with 10 ml scintillation fluid (MP Biochemicals). Radioactivity was counted after 24 h to allow for complete elution of [^{3}H]-iloprost into the scintillation fluid and to reduce chemiluminescence.

2.3. Determination of B_{max} and K_d

Values obtained from saturation binding experiments were entered into GraphPad Prism software and values for the number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated with non-linear regression by fitting a hyperbola directly to the saturation isotherm. Scatchard Plots were drawn to visualize the saturation binding data.

2.4. cAMP assays

Vehicle (saline) or 1 μM iloprost was added to the platelets prior to incubation for 5 min at room temperature. After incubation, chilled acidified ethanol (4 ml, 4 °C) was added to the platelet suspension. The suspension was centrifuged at 21,000 × *g* for 10 min at 4 °C. The supernatant was removed and dried under vacuum centrifugation. Samples were reconstituted in assay buffer and cAMP was measured using a cAMP Enzyme Immunoassay (GE Healthcare) in which cAMP values were normalized to 5×10^8 platelets. Platelet counts were performed in a hemacytometer.

2.5. Western blot analysis

Platelets in Western blot extraction buffer were sonicated briefly while on ice. After 20 min incubation on ice, the platelet lysate was centrifuged at 4 °C for 30 min 500 × *g*. Platelet lysates (10 μl) were aliquotted and stored at −80 °C until the day of use. Platelet lysates (50 μg protein) were solubilized in 2× Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl, pH 6.8, Sigma) and boiled for 5 min. Samples were chilled on ice for 5 min and loaded onto pre-cast 4–20% SDS-polyacrylamide gels (Pierce), resolved by electrophoresis, then transferred to polyvinyldiene difluoride (PVDF) membranes. PVDF membranes were blocked for 1 h at room temperature in Starting Block® (Thermo Scientific) containing 0.01% Tween-20 and incubated overnight (16 h) at 4 °C with a monoclonal antibody (1:200, Abnova) directed against the N-terminal portion of the IPR followed by washing and incubation for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, GE Healthcare). IPR protein expression was visualized using Super Signal West Pico chemiluminescent substrate (Pierce) on photographic film. PVDF membranes were then stripped of antibody using Restore® stripping buffer (Thermo Scientific), blocked for 30 min at room temperature in Starting Block® (Thermo Scientific) containing 0.01% Tween-20 and incubated for 1 h at room temperature with primary antibody for β-actin (1:5000, Sigma) followed by washing and incubation with a HRP-conjugated secondary antibody (1:5000, GE Healthcare). β-Actin protein expression was visualized by enhanced chemiluminescence (ECL, Pierce) on photographic film. The intensity of the chemiluminescent signals captured on photographic film was converted to pixel density using QuantiScan software. The pixel density of IPR protein expression was normalized to the pixel density of β-actin protein expression.

2.6. Statistical analysis

Statistical significance between groups was determined using an analysis of variance (ANOVA). In the event that the *F* ratio indicated that a change had occurred, a Fisher's Least Significant Difference (LSD) test was performed to identify individual differences between groups. Results are reported as the means ± the standard error (SE).

Linear regression analysis was performed using GB STAT software, and the Line of Best Fit was plotted.

3. Results

3.1. Characteristics of the subjects

Individuals with type 2 diabetes were identified by physicians at Saint Louis University in the Endocrinology Clinic. A patient history was collected for each individual including a detailed listing of current medications and the patient's age and gender. The subjects studied were healthy human volunteers (n = 17, 9 female, 8 male) and humans with type 2 diabetes (n = 15, 8 female, 7 male) with a mean age of 47 years (range 23–76 years) and 50 years (range 31–74 years), respectively. The average HbA1c of humans with type 2 diabetes in this study was 7.9 ± 0.4%. Patients with type 2 diabetes were treated with multiple drugs in various combinations including aspirin (n = 10), angiotensin converting enzyme inhibitors or angiotensin receptor blockers (n = 11), β-adrenergic receptor blockers (n = 5), oral hypoglycemic agents (n = 12), insulin (n = 13), lipid lowering drugs (n = 9), calcium channel blockers (n = 3) and diuretics (n = 3). The nature of the patients' illnesses precluded discontinuation of medications. Record keeping was in compliance with HIPAA (Health Insurance Portability and Accountability Act) regulations.

3.2. Platelets of humans with type 2 diabetes exhibit decreased saturation binding of ^{3}H-iloprost when compared to platelets of healthy humans

Saturation binding studies using increasing concentrations (10–100 nM) of [^{3}H]-iloprost demonstrated that specific binding with 100 nM [^{3}H]-iloprost was significantly decreased (P < 0.05) in