Chapter 18

EXPRESSION OF THE HETEROTRIMERIC G PROTEIN Gi AND ATP RELEASE ARE IMPAIRED IN ERYTHROCYTES OF HUMANS WITH DIABETES MELLITUS

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Abstract: Erythrocytes of humans have been reported to stimulate nitric oxide (NO) synthesis in the circulation as a consequence of their ability to release ATP in response to both mechanical deformation and exposure to reduced oxygen tension. It has been proposed that the ability of the erythrocyte to affect local vascular resistance permits it to participate in the regulation of blood flow such that oxygen delivery is matched with metabolic need. A signal transduction pathway that relates deformation and exposure to reduced oxygen tension to ATP release from human erythrocytes has been described. The heterotrimeric G protein, Gi, is a critical component of this pathway. Importantly, stimulation of Gi results in activation of adenylyl cyclase and ATP release from these cells. Recently, in a model of diabetes mellitus in rats, expression of Gi was reported to be decreased in the aorta. We report that expression of $G\alpha_{i2}$ is selectively decreased in erythrocytes of humans with type 2 diabetes (DM2) and that these erythrocytes fail to release ATP in response to incubation with mastoparan 7 (10 μM), an agent that activates Gi. These results provide support for the hypothesis that ATP release from erythrocytes of humans with DM2 is impaired and this defect in erythrocyte physiology could contribute to the vascular disease associated with this clinical condition.

Key Words: mastoparan 7, heterotrimeric G proteins, western analysis

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INTRODUCTION

Previously, we reported that human erythrocytes release ATP in response to both mechanical deformation (28,29,30) and exposure to reduced oxygen tension (3,8,9). In the vasculature, P_{2y} purinergic receptors are found on endothelial cells (5,7,16). Binding of ATP to these receptors results in the synthesis of NO (5,16). We have proposed that ATP released from the erythrocyte acts locally on endothelial cells to evoke NO release which, in turn, affects vascular caliber permitting the erythrocyte to participate in local regulation of the circulation. Indeed, the erythrocyte has been suggested to be an important determinant of the matching of oxygen supply with tissue metabolic demand (8,9,11).

The fact that ATP does not readily cross cell membranes suggests that its release from erythrocytes requires a specific mechanism. We reported that, in human erythrocytes, Gs (22) and Gi (21,23), adenylyl cyclase (30), cyclic AMP-dependent protein kinase (PKA) (30) and the cystic fibrosis transmembrane conductance regulator (CFTR) (27) are components of a signal-transduction pathway that relates physiological stimuli such as mechanical deformation or exposure to reduced oxygen tension to ATP release (Figure 1).

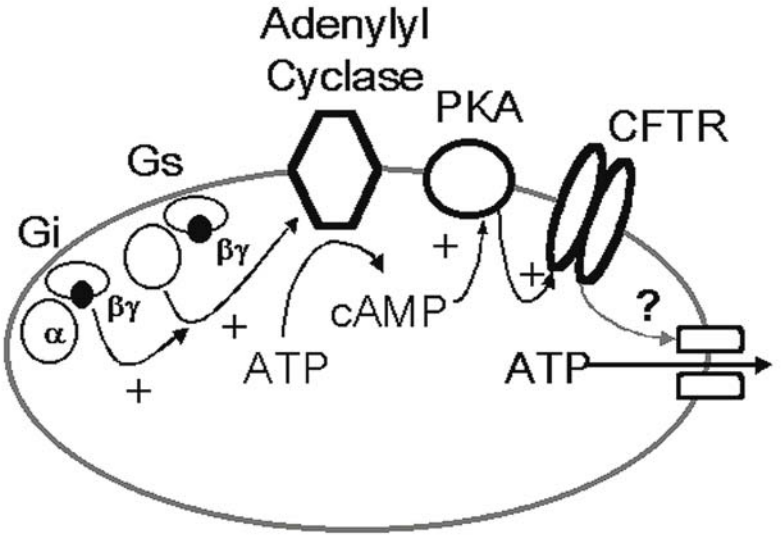

Figure 1. Proposed pathway for regulated ATP release from human erythrocytes. Abbreviations: Gs and Gi - heterotrimeric G proteins; ATP - adenosine triphosphate; cAMP - 3'5'-cyclic-adenosine monophosphate; PKA - protein kinase A; CFTR - cystic fibrosis transmembrane conductance regulator**; ?** - a yet unidentified conduit for ATP release; + - stimulates.

Recently, altered expression of one of the components of this pathway, Gi, has been reported to occur in an animal model of diabetes mellitus (12). In rats made diabetic with streptozotocin, significant reductions in the expression of the heterotrimeric G-proteins, $G\alpha i_2$ and $G\alpha i_3$ were detected in the aorta (12). This finding is of particular interest as we have demonstrated that Gi is a necessary component of a signal-transduction pathway for ATP release from erythrocytes of humans (21,23) in response to mechanical deformation

and exposure to reduced oxygen tension. Importantly, Gi activation results in stimulation of adenylyl cyclase and ATP release from these erythrocytes (21,23).

The finding that activation of Gi is a critical component of a signal transduction pathway for ATP release from erythrocytes in response to physiological stimuli, coupled with reports that expression of this G protein is reduced in experimental diabetes, suggested to us that erythrocytes of humans with diabetes might have a defect in ATP release from erythrocytes that could, ultimately, lead to a decreased stimulus for endogenous NO synthesis. Here, we investigated the hypothesis that expression of the heterotrimeric G protein, Gi, is reduced in erythrocytes of humans with type 2 diabetes (DM2) and that this defect is associated with decreased ATP release in response to an agent that activates that G protein, mastoparan 7 (MAS 7) (15,21,23).

METHODS

Generation of washed erythrocytes: Blood was obtained from healthy humans as well as humans with type 2 diabetes mellitus (DM2) by venipuncture (antecubital fossa). Blood was collected into a heparinized syringe and centrifuged at 500 x g at 4°C for 10 min. The plasma and buffy coat were discarded. Erythrocytes were resuspended and washed x 3 in buffer (in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl 1.2 $MgSO_4$, 21.0 tris(hydroxymethyl)amino methane, 5.5 glucose with 0.5% bovine serum albumin (final pH 7.4)). Cells were prepared on the day of use.

ATP assay: ATP was measured by the luciferin-luciferase technique (3,21-23,27-30) in which the amount of light generated by the reaction of ATP with firefly tail extract is dependent on ATP concentration. Sensitivity was augmented by addition of synthetic D-luciferin. A 200 µl sample of erythrocyte-containing solution (or ATP standard) was injected into a cuvette containing 100 µl firefly tail extract (10 mg/ ml distilled water, FLE 50, Sigma, St. Louis, MO) and 100 µl of a solution of synthetic D-luciferin (5 mg/10 ml distilled water, Sigma, St. Louis, MO). The peak light efflux was determined using a luminometer (Turner Designs, Sunnyvale, CA, model 20/20). A standard curve was obtained on the day of each experiment. Values for ATP were normalized to an erythrocyte count of 4 x 10^5 cells/mm^3.

Measurement of hemoglobin: To exclude the possibility that concentrations of extracellular ATP represent that released by the lysis of erythrocytes, after ATP determinations, erythrocyte suspensions were centrifuged at 500 x g at 4°C for 10 min and the presence of hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm (33). The assays for ATP and hemoglobin have similar sensitivities such that an increase in ATP, if unaccompanied by an increase in hemoglobin, represents active ATP release from erythrocytes and is not the result of cell lysis. If increases in hemoglobin were detected, the study was excluded.

Preparation of erythrocyte membranes: Erythrocyte membranes were prepared as follows. All lytic steps were conducted at 4°C. Washed RBCs (2 ml) were added to 200 ml of hypotonic buffer (in mM; 5 Tris-HCl, 2 EDTA with pH adjusted to 7.4) and stirred vigorously for 20 min. The mixture was centrifuged at 23,300 x g for 15 min. The super-

natant was discarded and the membranes were re-suspended in buffer. The mixture was centrifuged at 23,300 x g for 15 min and the supernatant discarded. The membranes were pooled, re-suspended in buffer, and centrifuged again at 23,300 x g for 15 min. The protein concentration was determined with the BCA Protein Assay (Pierce).

Identification of the α subunit of heterotrimeric G proteins in erythrocyte membranes: Erythrocyte membranes were solubilized in SDS sample buffer (0.277 M sodium dodecyl sulfate (SDS), 60% glycerol, 0.4 M dithiothreitol, 0.25 M Tris HCl, and 0.004% bromophenol blue) and heated (5 min, 100°C) before loading onto a precast 4-20% gradient Tris-HCl Ready Gel (Bio-Rad). Gels were subjected to electrophoresis at 150 V for 1.5 h with buffer containing 25 mM Tris, 192 mM glycine and 0.1% w/v SDS, pH 8.3. After electrophoresis, proteins were transferred for 1 h on ice onto a polyvinylidene difluoride (PVDF) membrane with transfer buffer (25 mM Tris and 192 mM glycine with 20% v/v methanol at pH 8.3) at 100 V. PVDF membranes were blocked (overnight, 4 °C) with 5% non-fat dry milk in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), then incubated with antibodies directed against the alpha subunit of Gs, (rabbit polyclonal), Gi_2 (mouse monoclonal), and Gi_3 (rabbit polyclonal) (Biomol). In addition, membranes were incubated with antibody directed against ß-actin, a structural protein in erythrocytes. PVDF membranes were then incubated (4 h, 25 °C) with donkey anti-rabbit or sheep anti-mouse IgG linked to horseradish peroxidase (Amersham Biosciences) as the secondary antibody in 1% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween 20. After labeling with the secondary antibody, PVDF membranes were exposed to enhanced chemiluminescence using ECL (Amersham Biosciences).

Determination of relative amounts of G protein α subunits present in erythrocyte membranes: Amounts of membrane protein loaded on the gels were standardized by determination of the protein content of each preparation prior to loading and by determination of the amount of ß-actin present in each sample on separate gels with protein diluted 5 fold. Amounts of G protein in individual membrane preparations were calculated as the ratio of G protein to β-actin . Values measured in humans with DM2 are expressed as the percentage of that detected in the membranes of healthy humans run on the same gel. All studies were performed in duplicate.

Study of Patients with Diabetes Mellitus Type 2 (DM2): Patients with diabetes mellitus type 2 (DM2) were identified by physicians in the Endocrine Clinic at Saint Louis University. A history form was completed for each individual studied (both healthy volunteers and humans with DM2). The information obtained included a medication history, a detailed listing of all medications and age, as well as the most recent HbA1c level (values within four weeks of blood removal). The mean age of healthy humans and humans with DM2 was 40±5 and 51±5 respectively. Patients with DM2 (n=9) were treated with insulin (n=8), lipid lowering agents (n=8) oral hypoglycemic agents(n=6), anti-platelet agents (n=6), diuretics (n=5), β blockers (n=5), angiotensin converting enzyme inhibitors (n=5) and calcium channel blockers (n=3). All record keeping was in strict compliance with HIPPA regulations.

Incubation of erythrocytes with MAS7: After washing, erythrocytes were diluted with wash buffer (see above) to achieve a hematocrit of 20%. Erythrocytes of healthy humans or humans with DM2 (HbA1c values of 8 or greater, range 8.1 to 11.8) were incubated with either mastoparan 7 (MAS 7, activator of Gi, 10 μM) or its vehicle (saline). ATP release was measured at 5, 10 and 15 minutes after addition of MAS 7 or buffer. ATP was measured by adding 200 μl of erythrocyte suspension diluted 250 fold to the luciferin/luciferase mixture. The amount of free hemoglobin in each sample was measured.

Statistical methods: Statistical significance between experimental periods was determined with an analysis of variance. In the event that the *F* ratio indicated that changes had occurred, a least significant difference test was used to identify individual differences. *P* values of 0.05 or less were considered statistically significant. Results are reported as means ± SE.

RESULTS

Quantification of heterotrimeric G protein α subunits in erythrocyte membranes: Erythrocyte membranes of humans with DM2 were found to contain amounts of Gsα (n=4, HbA1c=12.3±1.8) and $G\alpha i_3$ (n=5, HbA1c=12.2±1.4) that were not different from those found in heathy humans (n=6 and 5, respectively) (Figure 2). In contrast, expression of $G\alpha i_2$ (n=5, HbA1c=12.2±1.4) was reduced to 56±2 % of that found in healthy humans (n=8) (Figures 2 and 3).

ATP release from erythrocytes in response to MAS 7: Incubation of erythrocytes of healthy humans with 10 μM MAS 7 resulted in 7 ± 1 fold increase in ATP release (n=8, *P*<0.01) (figure 4). In contrast, incubation of erythrocytes of humans with DM2 (HbA1c= 10.1± 0.7, n=5) with MAS 7 did not result in increased ATP release (Figure 4).

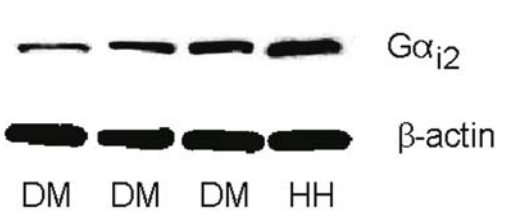

Figure 2: Measurement of the heterotrimeric G protein $G\alpha i_2$ and β-actin in erythrocyte membranes in a healthy human (HH) and humans with diabetes mellitus type 2 (DM). Membranes were prepared and the proteins were resolved using a 4-20% gradient TRIS-HCl pre-cast gel. Protein was transferred to a PVDF membrane and incubated with a mouse monoclonal anti-$G\alpha i_2$ antibody.

DISCUSSION

In humans with diabetes mellitus (DM), cardiovascular disease accounts for 66% of deaths (14,20). The most prevalent form of human DM (90-95% of individuals) is classified as type 2 (DM2) (20). DM2 is characterized by peripheral insulin resistance with an insulin secretory defect that varies in severity. It occurs typically in individuals over

40 years of age who have a family history of diabetes. Over 90% of these individuals are obese. Patients with DM2 retain the ability to secrete some endogenous insulin, therefore, they do not develop diabetic ketoacidosis. Thus, these individuals may require insulin and/or oral hypoglycemic agents to control blood sugar levels, but do not depend on them to prevent ketoacidosis.

The observation that vascular reactivity is altered in humans with DM2 is widely accepted (14,20). Indeed, both endothelium-dependent and -independent vasodilation is impaired in humans with DM2 (1,19,34,35). Impaired vasodilation in DM2 has been attributed to decreased NO synthesis (34), increased NO degradation (1) and/or to abnormalities in the vascular smooth muscle (35). Mechanism(s) notwithstanding, NO-mediated vasodilation is reduced in humans with DM2. It is possible that, in addition to the endothelium, other sources of endogenous NO may be compromised in these individuals. In addition, changes in erythrocyte physiology have also been reported to be present in humans with DM.

Erythrocytes of patients with DM have been reported to have reduced glutathione concentration (6) although this observation has not been confirmed by others (17). However, it is agreed that the oxidant stress to which erythrocytes are exposed is increased in a high glucose environment (6,17). It has been hypothesized that this oxidant stress leads to increased glycation of erythrocyte proteins (4). Glycated hemoglobin (HbA1c) has been accepted as a measure of the degree of glycemic control in patients with diabetes such that the lower the HbA1c level, the better the glycemic control (26,32). Indeed, HbA1c levels correlate with diabetic complications (17,32), i.e., better glycemic control is associated with reduced complications.

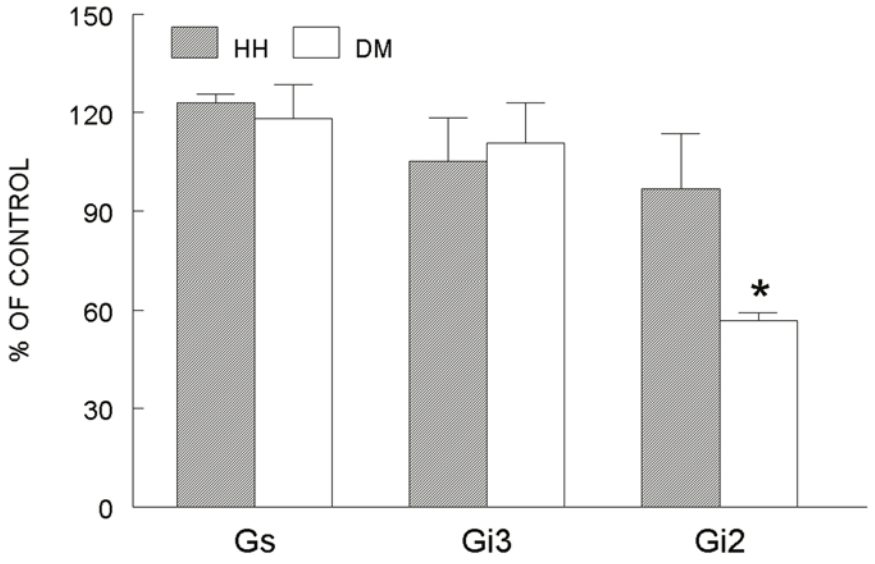

Figure 3. Expression of heterotrimeric G protein α subunits in erythrocyte membranes of healthy humans (HH, cross hatched bars) and humans with diabetes mellitus type 2 (DM, open bars). Numbers of humans studied are: Gs, 5 HH and 4 DM; Gi3, 4 HH and 5 DM and Gi2, 7 HH and 5 DM. Values are calculated as the % of the amount of G protein α subunit found in a control human erythrocyte preparation. Values are the mean ± SEM. *, $p<0.05$ compared to all other groups.

In addition to glycated hemoglobin, several properties of the membranes of RBCs of patients with diabetes differ from those of healthy humans (13,24,25). For example, exposure of erythrocytes to increased concentrations of D-glucose results in altered conductivity of the cell membrane (13). Erythrocytes of diabetics have also been reported to be less deformable that those of healthy humans (18). More important to this study, erythrocytes of patients with diabetes demonstrate a defect in ATP release in response to osmotic stress (24) and contain increased amounts of ATP (24), the latter finding possibly reflecting a failure of those mechanisms that result in ATP release. Here we investigated the hypothesis that such a defect in ATP release from erythrocytes of humans with DM2 is the result of the failure of a component or components of a signal transduction pathway for ATP release.

As stated above, we have defined a signal transduction pathway that relates physiological stimuli to ATP release from erythrocytes of rabbits and humans (figure 1). Recently, altered expression of one of the components of this pathway, Gi, has been reported in an animal model of diabetes (12). Thus, in rats made diabetic with streptozotocin, significant reductions in the expression of the heterotrimeric G-proteins, Gαi2 and Gαi3 were detected in the aorta (12). This finding is of particular interest in that we have demonstrated previously that Gi is a necessary component of a signal-transduction pathway for deformation- and reduced oxygen tension-induced ATP release from erythrocytes of healthy humans (21,23).

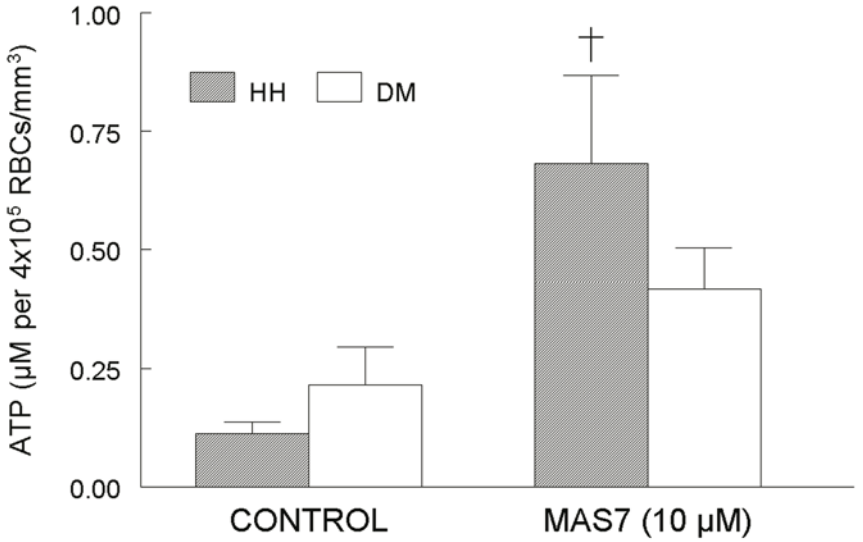

Figure 4. Effect of mastoparan 7 (MAS7) on ATP release from erythrocytes of healthy humans (HH, n=8) and humans with diabetes mellitus type 2 (DM, n=5). Washed erythrocytes were incubated with MAS7 or its vehicle (saline, control). ATP release was measured at 5, 10 and 15 min after MAS7 and the maximal response is reported. Values are the mean ± SEM. †, $p<0.05$ compared to respective control.

It is now recognized that, in addition to the α subunits of heterotrimeric G proteins, the βγ subunit is capable of activating at least three of eight membrane associated isoforms of adenylyl cyclase, specifically, subtypes II, IV and VII (table 1) (10,31). The heterotri-

meric G proteins most clearly associated with this property are of the Gi/o subclass (31). The ability of the βγ subunit to activate adenylyl cyclase subtypes II, IV and VII has been shown to reside with the β component of that dimer, of which five types have been defined (31). Of five β subunit types that have been identified, the ability to stimulate adenylyl cyclase has been associated with types 1, 2, 3 and 4, but not type 5 (2). We have reported that β subunits 1,2,3,and 4 are components of the erythrocyte membranes of humans (28). Thus, G proteins of both the Gs and Gi subclasses, as well as β subunits capable of stimulating adenylyl cyclase, are present in human erythrocytes.

The finding that activation of Gi is a critical component of a signal transduction pathway for ATP release from erythrocytes in response to physiological stimuli, coupled with the finding that expression of this G protein is reduced in the erythrocyte membranes of humans with DM2, suggests that erythrocytes of humans with DM2 could have a defect in ATP release from erythrocytes that leads to a decreased stimulus for endogenous NO synthesis. Thus, we conclude that defects in the ability of erythrocytes to release ATP in response to physiological stimuli could contribute to vascular disease in humans with DM2.

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