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Regulators of G protein signaling are up-regulated in aspirin-resistant platelets from patients with metabolic syndrome

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G protein-coupled receptor signaling plays a crucial role in platelet function. Regulators of G protein signaling (RGSs), which accelerate the deactivation of G protein signaling, are expressed in platelets. However, RGS expression has not been studied in the context of aspirin resistance. We compared RGS mRNA levels in platelets from 39 aspirin-resistant patients and 50 aspirin-sensitive patients with metabolic syndrome. Although there were no clinical differences between the two groups, transcripts of RGS2, RGS10, and RGS18 were significantly higher in aspirin-resistant patients than in aspirin-sensitive patients. This study is the first to demonstrate that RGS transcripts are elevated in aspirin-resistant platelets from patients with metabolic syndrome.

1. Introduction

Acetylsalicylic acid (aspirin) is the most commonly used antiplatelet drug worldwide in both primary and secondary prevention of cardiovascular disease (ISIS-2 Collaborative Group 1988; Antithrombotic Trialists' Collaboration 2002). High-risk vascular patients treated with aspirin have a 34% reduction in nonfatal myocardial infarction, 25% reduction in nonfatal stroke, and 18% reduction in all-cause mortality (Antithrombotic Trialists' Collaboration 2002). Aspirin acts by irreversibly inhibiting the cyclooxygenase-1 enzyme to reduce thromboxane A2 (TXA2), a potent vasoconstrictor and activator of platelet aggregation. However, the antiplatelet effects of aspirin may not be equal in all individuals; a proportion of patients under aspirin suffer recurrent thromboembolic vascular events, sometimes referred to as aspirin resistance. In some cases failure to respond to aspirin may be caused by an inadequate primary pharmacological effect. Gum et al. defined aspirin resistance as a mean aggregation of $\geq$ 70% with 10 μmol/L adenosine diphosphate (ADP) or $\geq$ 20% with 0.5 mg/mL arachidonic acid (Gum et al. 2001, 2003).

Platelet activation and subsequent recruitment of additional platelets is strongly dependent on intracellular signaling pathways that are mainly induced *via* diverse G protein-coupled receptors (GPCRs). Most platelet agonists (such as thrombin, ADP, and TXA2) activate GPCRs that mainly signal through activation of their specific G protein to induce calcium release and inhibition of adenylyl cylases, leading to platelet activation (Offermanns 2006). Regulators of G protein signaling (RGSs) are intracellular signaling regulators that bind activated G protein α subunits (Gα) and increase their intrinsic GTPase activity via their common RGS homology domain, thus terminating GPCR signaling (Watson et al. 1996). The identification of mRNAs for RGS 1, 2, 3, 6, 9, 10, 16, 18 and 19 in human platelets (Bodor et al. 2004) and RGS 2, 3, 5, 6, 10, 14, 16 and 18 transcripts in rat platelets (Kim et al. 2006), suggests that RGS molecules may be modifiers of the G protein signaling cascade. Yet the role of RGS proteins in platelet function under pathological conditions remains poorly understood.

Following on from a cross-sectional study for aspirin resistance in a cohort with metabolic syndrome (Liu et al. 2008), this study aimed to compare the expressions of regulators of G protein signaling in the platelets of aspirin resistant and aspirin sensitive patients.

2. Investigations, results and discussion

Aspirin resistance was documented in 39 out of 221 patients (17.6%) with metabolic syndrome. We used the platelets from all 39 aspirin resistant patients and 50 randomly selected aspirin sensitive patients. Clinical features for these patients are shown in the Table. The platelet aggregation rate was 9.3 ± 7.8% in sensitive patients, and 32.5 ± 15.7% in resistant patients ($P < 0.05$). The two groups did not differ significantly with respect to age, sex, blood pressure, fasting plasma glucose, lipid profile, body mass index, waist circumference, and history of smoking, heart attack or stroke. The gene expressions of RGS 2, 10 and 18 were all significantly higher in the aspirin resistant group compared to the aspirin sensitive group (Fig.).

It has been demonstrated that RGS proteins can be robust inhibits of platelet activation. Mutant mice with a G185S substitution in their Gα subunit were generated (Signarvic et al. 2010). This mutation makes the Gα subunit resistant to accelerated turn-off by all RGS proteins but does not change its coupling to GPCRs (Huang et al. 2006). These mice showed enhanced platelet reac-

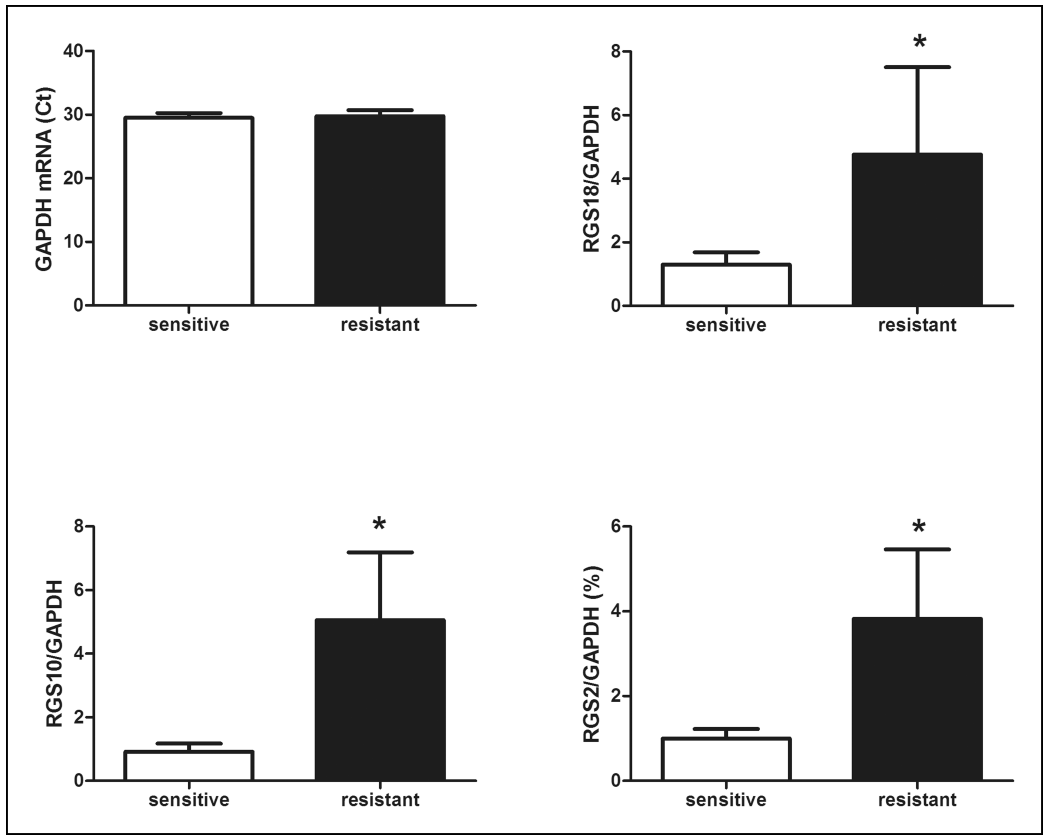

Fig.: Gene expression of RGSs in platelets. RGS2, RGS10, and RGS18 to GAPDH transcription ratios in platelets were compared between aspirin resistant group (n = 39) and aspirin sensitive group (n = 50) in patients with metabolic syndrome. Mean ± SEM; *, $P < 0.05$ *vs.* aspirin sensitive group. RGS: regulator of G protein signaling, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

tivity to different platelet agonists in aggregation studies and an increased thrombus formation at sites of vascular injury (Signarvic et al. 2010). Additionally, in patients with a heterozygous RGS2 gain-of-function mutation (G23D), the mutation results in increased expression of larger RGS2 isoforms that interact strongly with adenylyl cyclase to inhibit its function, leading to decreased Gα activity in platelets. The study also suggests a negative regulatory function of RGS2 in platelet activation (Noe et al. 2010).

Table: Clinical features of participants by platelet function

	Aspirin sensitive (n = 50)	Aspirin resistant (n = 39)
Platelet aggregation rate, %	9.3 ± 7.8%	32.5 ± 15.7% *
Age, years	63 ± 10	64 ± 9
Sex, Male/Female	18/27	15/24
Smoking, %	8 (16%)	6 (15.4%)
Stroke, %	4 (8%)	4 (10.3%)
Heart attack, %	3 (6%)	5 (12.8%)
Waist circumference, cm	89.7 ± 9.8	90.6 ± 9.0
BMI, kg/m^2	26.8 ± 4.2	26.5 ± 3.4
Fasting plasma glucose, mmol/L	8.5 ± 2.7	8.5 ± 3.0
Systolic blood pressure, mmHg	132 ± 13	135 ± 22
Diastolic blood pressure, mmHg	80 ± 10	79 ± 11
TG, mmol/L	3.4 ± 2.3	3.3 ± 2.4
HDL, mmol/L	1.3 ± 0.3	1.2 ± 0.3
TC, mmol/L	5.5 ± 1.1	5.5 ± 1.0

Mean ± SD (except triglyceride is geometric mean ± SD). *, $P < 0.05$ *vs.* aspirin sensitive group by an unpaired t test or Fisher's exact test. BMI: body mass index, HbA1c: glycosylated hemoglobin A1c, TG: triglyceride, HDL: high- density lipoprotein, TC: total cholesterol.

In the current study, we first demonstrated that in patients of metabolic syndrome the platelet RGS mRNAs were expressed at significantly higher levels with aspirin resistance than with aspirin sensitiveness; further indication of the importance of RGSs in platelet function. Although there were significantly elevated RGSs transcript levels when arachidonic acid was used to activate the platelets via its GPCR, the aggregate rate was higher in the aspirin resistant group than that in aspirin sensitive group. This may indicate potent activation of GPCRs or over-sensitive GPCRs are involved in aspirin resistance in metabolic syndrome, which could not be effectively terminated by the elevated RGSs. Another possibility might be that the RGSs are functionally compromised because of an abnormal phosphorylation state. RGS18 was originally discovered as a potential new mediator of G protein signaling in human platelets (Gagnon et al. 2002), and was shown to have the highest mRNA expression compared with the other RGS proteins (Bodor et al. 2004; Kim et al. 2006). Moreover, the 14-3-3 protein binds to the phosphorylated serines 49 and 218 of RGS18 (Gegenbauer et al. 2012). Platelet activation by thrombin, TXA2 or ADP stimulates the association of 14-3-3 and RGS18, probably by increasing the phosphorylation of serine 49. In contrast, treatment of platelets with prostacyclin and nitric oxide induces the phosphorylation of serine 216 of RGS18 and the detachment of 14-3-3. In addition, 14-3-3–deficient RGS18 has been shown to be more active than 14-3-3–bound RGS18, leading to a more pronounced platelet inhibition (Gegenbauer et al. 2012). RGS18 modifies platelet signaling as part of a heterotrimer complex consisting of RGS18, spinophilin (SPL) and the tyrosine phosphatase (SHP-1) (Ma et al. 2012). In resting platelets, the complex prevents RGS18 binding with Gα subunit and executing its function as a negative regulator. However, once platelets become activated, SHP-1 will

be activated leading to a decay of the complex. This dissociation leads to a translocation of RGS18 to the G protein, where it will down-regulate GPCR signaling and inhibit platelet activation (Ma et al. 2012). SPL-deficient mice unable to form stable SPL/SHP-1/RGS18 complexes have continuous RGS18 activity, impaired platelet aggregation and prolonged carotid artery occlusion time (Ma et al. 2012).

In conclusion, our study showed that platelet RGS mRNAs were expressed significantly higher in metabolic syndrome patients with aspirin resistance than in aspirin sensitive patients. This may indicate that either potent activation of GPCRs, oversensitive GPCRs or functional inhibition of RGSs is involved in this condition. However, the study was limited in that we could not determine the protein expression levels and platelet functional responses with other agonists in the cohort.

3. Experimental

3.1. Patients

We have conducted a cross-sectional study to investigate the profile and prevalence of aspirin resistance in patients with metabolic syndrome (Liu et al. 2008). The study was in adherence to the Declaration of Helsinki and was approved by institutional review board. The study population consisted of 221 consecutive patients recruited from the Shougang community between May and July 2005 after obtaining a written informed consent. All patients reached the NCEP ATP III criteria modified by racial variety and met at least 3 of the following criteria: abdominal obesity (waist circumference > 85 cm in men and > 80 cm in women), high serum triglycerides (>1.7 mol/L), low HDL cholesterol (< 1.0 mmol/L in men and < 1.3 mmol/L in women), high blood pressure (≥ 130/85 mmHg) and high fasting plasma glucose (≥ 6.1 mmoI/L). Patients with liver or renal dysfunction, malignant tumor, allergy or intolerance to aspirin, administration of warfarin, antiplatelet or non-steroidal anti-inflammatory agents, or platelet count < 100×10^9/L or > 500×10^9/L were excluded.

3.2. Study protocol

In brief, all patients received aspirin (200 mg daily) for 10 days. Fasting blood samples were collected with 3.8% sodium citrate between 1-3 h after last dosing. Conventional hematological measurement, optical platelet aggregation and biochemical assessments were performed. Platelets were isolated with precaution to minimize leukocyte contamination as described previously (Liu et al. 2008). Aspirin resistance was defined as a mean aggregation ≥20% with 0.5 mg/mL arachidonic acid.

3.3. Gene expression

Total RNA was extracted from the stored platelets using Trizol (Life Technologies, Gaithersburg, MD, USA). The reverse transcription polymerase chain reaction (PCR) was performed using TOYOBO ReverTra Ace-α-RT-PCR kit. The expression of selected mRNAs was measured by qRT-PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems). Expression of the mRNAs was normalized to that of the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. PCR reactions were performed in 25 μl of buffer containing 1 μl of cDNA, SYBR Green Master Mix (Applied Biosystems) and 5 pmol of sequence–specific primers: RGS2 (forward primer, 5'-CTG CAG ACC CAT GGA CAA GA-3'; reverse primer, 5'-TTG GGC TTC CCA GGA GTA GA-3'), RGS10 (forward primer, 5'- GTC CAG CAA GGC CTC ATC AC-3'; reverse primer, 5'- CGG CTG TAG CTG TCG TAC TTCA-3'), RGS18 (forward primer, 5'- AGT CTT CTT GTG CAG AAA CCT GAGT-3'; reverse primer, 5'- ATC TCT ATG GGA AAG CAG TTT GTCA-3'), or GAPDH (forward primer, 5'- AAT CCC ATC ACC ATC TTC CA-3'; reverse primer, 5'- TGG ACT CCA CGA CGT ACT CA-3'). Thermal cycling conditions consisted of a preincubation step for 2 min at 50 °C, then denaturation for 10 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 1 min at 60 °C. All qRT-PCR reactions were performed in triplicate.

3.4. Statistical analysis

Statistical analyses were performed using SPSS version 17. An unpaired t test or Fisher's exact test was used to compare variables between the 2 groups. A P value < 0.05 was considered statistically significant. Triglyceride levels were log-transformed and expressed as geometric mean ± SD. Gene expressions were expressed as mean ± SEM and all other scale variables were expressed as mean ± SD.

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