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Enhanced epinephrine-induced platelet aggregation in individuals carrying the G protein β3 subunit 825T allele

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Abstract The 825T allele of a common C825T polymorphism in the gene encoding the β3 subunit of heterotrimeric G proteins is associated with enhanced activation of pertussis toxin (PTX)-sensitive G proteins. We investigated responses of human platelets upon stimulation with epinephrine, which activates PTX-sensitive G proteins, and with agonists which activate additionally, or exclusively PTX-insensitive pathways. Slopes and maximum of the secondary aggregation were significantly enhanced in platelets from 825T allele carriers after epinephrine, and after combined epinephrine/ADP. This effect was more pronounced after inhibition of the cyclooxygenase-2 pathway by acetylsalicylic acid. This phenomenon appeared independent of platelet secretion, or inhibition of the adenylyl cyclase.

Key words: Calcium; Human; Gene; Platelet; G protein; Epinephrine

1. Introduction

The G protein α subunits which are activated in human platelets by common agonists have been described: vasopressin (VP) induces platelet aggregation via the V_1 receptor involving the pertussis toxin (PTX)-insensitive Gαq [1]. The thromboxane A_2 (TXA_2) receptor agonist U46619 activates Gα12, Gα13, Gαq but not the PTX-sensitive Gαi [2,3]. Thrombin receptor-activating peptide (TRAP) activates Gα12, Gα13, Gαq, and Gαi [2,4]. ADP activates Gαq and Gαi [5]. Using a knockout mouse model, Offermanns et al. documented that Gαq is essential for aggregation in response to TXA_2, thrombin, and ADP [6], whereas Gα12/13 proteins mediate the platelet shape change reaction [7]. In contrast, epinephrine-induced platelet aggregation is considered to be exclusively mediated by Gαi2 after activation of the α_{2A} adrenoceptor [8].

We have described a common C825T polymorphism in the gene *GNB3*, encoding the ubiquitously expressed β3 subunit of heterotrimeric G proteins [9]. The 825T allele is associated with alternative splicing of the gene and the generation of a truncated, but functionally active β subunit referred to as Gβ3s. Gβ3s was demonstrated to be expressed in platelets of 825T allele carriers by means of Western blot analysis [9], and the 825T allele is predictive of enhanced signal transduction via PTX-sensitive G proteins [9–11].

Given the activation of PTX-sensitive G proteins by epinephrine together with the observation that the α_{2A} adrenoceptor activates G protein heterotrimers containing Gβ3 [12], we hypothesized that epinephrine-mediated platelet aggregation should be enhanced in *GNB3* 825T allele carriers. In contrast, platelet activation evoked by thrombin, ADP, or U46619, TRAP and VP may not be influenced by the allele status at the 825 positions of *GNB3*.

2. Materials and methods

Only healthy, non-smoking, male individuals between 18 and 40 years of age with no medication known to interfere with platelet function for at least 15 days were enrolled. Fura 2-acetoxymethylester and the thromboxane agonist U46619 were purchased from Calbiochem (La Jolla, CA, USA). Thrombin receptor agonist (TRAP) and bovine [Arg^8]-VP were obtained from Calbiochem–Novabiochem (San Diego, CA, USA). Epinephrine (Suprarenin™) was purchased from Hoechst AG (Frankfurt, Germany). Acetylsalicylic acid, forskolin, ADP, and thrombin were obtained from Sigma (Deisenhofen, Germany).

Platelet-rich plasma and platelet-poor plasma (PPP) were prepared as previously described [13]. All measurements were conducted within 3 h after preparation of platelets. Aggregation was monitored over 10 min using a single channel aggregometer (Chrono-Log Model 500-CA, Haverton, PA, USA) with continuous stirring at 900 rpm, prewarmed at 37°C, and calibrated before each single measurement. Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined in Fura 2-loaded platelets as described [13], using a spectrofluorometer (LS-5B, Perkin Elmer Corporation, Germany). Platelet overall secretion was estimated by analysis of the expression of P-selectin (CD62) as a marker of α degranulation, and GP53 (CD63) as a marker of dense granule secretion by use of an EPICS XL flow cytometer (Beckman Coulter, USA) mainly as described [14]. Intracellular cAMP concentrations were measured by a radioimmunoassay (Amersham Pharmacia Biotech, Freiburg, Germany) following the manufacturer's recommendations. Genotype analysis was performed as described [9]. The mean of triplicate measurements was calculated and groups were compared using Student's *t*-test for continuous variables. Differences were regarded significant at $P < 0.05$.

3. Results and discussion

Addition of epinephrine evoked the typical biphasic aggregation pattern seen in citrated plasma, the first phase representing primary (reversible) aggregation and the second phase representing secondary aggregation, which is initiated by the

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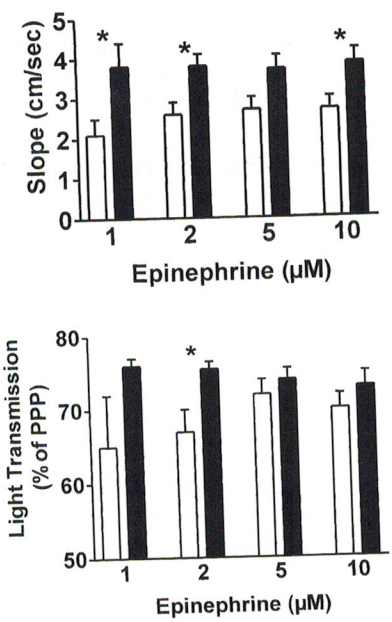

Fig. 1. Slope and maximum of the secondary aggregation after epinephrine. Light transmission is given as % PPP. ■ *GNB3* 825 TT and TC ($n=16$); □ *GNB3* 825 CC ($n=18$); $^*P<0.05$.

release of prostaglandin endoperoxide and platelet granule contents [15]. Primary aggregation was virtually identical in C825 and 825T allele carriers (data not shown). The slope of the secondary aggregation was approximately 2-fold increased in 825T allele carriers following stimulation with 1, 2, and 10 μM epinephrine, and the total extent of secondary aggregation was significantly higher at 1 and 2 μM epinephrine (Fig. 1). Epinephrine is known as a weak platelet agonist, which is capable to enhance the response to other agonists such as ADP, even in concentrations that usually evoke no, or only slight aggregatory responses [16]. After the combination of 0.5

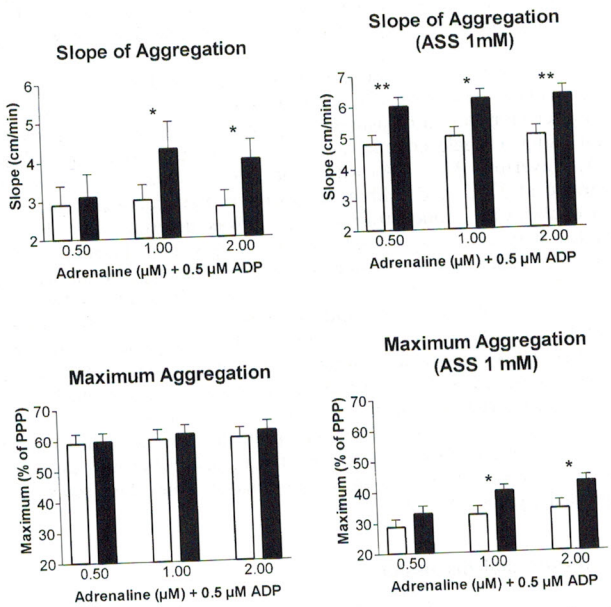

Fig. 2. Slope and maximum of the secondary aggregation after the combination of epinephrine and ADP before and after incubation with 1 mM acetylsalicylic acid. Light transmission is given as % of PPP. ■ *GNB3* 825 TT and TC ($n=16$); □ *GNB3* 825 CC ($n=18$); $P<0.05$.

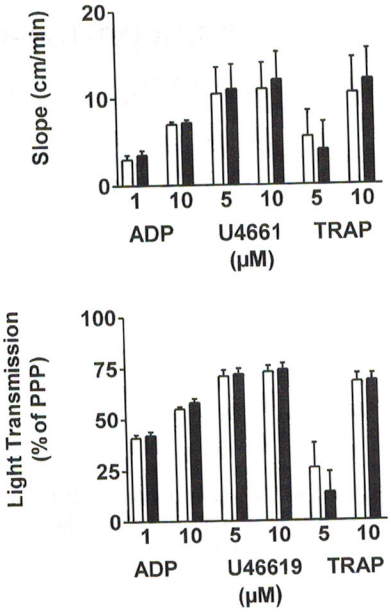

Fig. 3. Slope of aggregation and maximum of aggregation after stimulation with ADP, U46619, and TRAP. Light transmission is given as % of PPP. ■ *GNB3* 825 TT and TC ($n=16$); □ *GNB3* 825 CC ($n=18$).

μM ADP with low doses of epinephrine, the slope of the aggregatory response was significantly enhanced in carriers of an 825T allele at *GNB3*, however, the maximum response remained unchanged (Fig. 2). Most interestingly, the inhibition of the cyclooxygenase-2 pathway by acetylsalicylic acid leads to an even more pronounced and clearly significant difference between the genotypes (Fig. 2).

In contrast, after addition of ADP, U46619 and TRAP, the maximal slopes of the increase of light transmission as well as the maximal amplitudes of the reactions were not significantly different between genotypes at *GNB3* (Fig. 3).

Platelets from 825T allele carriers displayed also significantly higher basal values of $[Ca^{2+}]_i$ compared to platelets from homozygous C825 allele carriers (133 ± 17.4 nM versus 92 ± 9.9 nM; $P<0.05$; Fig. 4). This finding is interesting, since an increased basal $[Ca^{2+}]_i$ has repeatedly been found in cells from hypertensive individuals [17] and, therefore, keeps in line with the reported association of the 825T allele with hypertension [8,18,19]. This difference in $[Ca^{2+}]_i$ disappeared after agonist stimulation, and might, therefore, reflect a certain rate of pre-stimulation by e.g. enhanced sympathetic tone in these individuals.

These findings support our hypothesis that the 825T allele is a marker of enhanced signal transduction via pathways in-

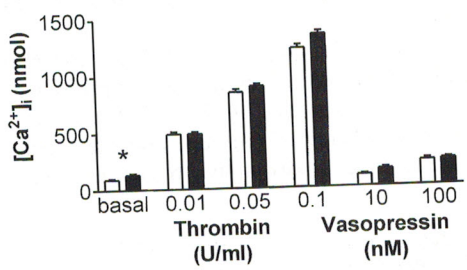

Fig. 4. Changes of $[Ca^{2+}]_i$ after thrombin and VP. ■ *GNB3* 825 TT and TC ($n=16$); □ *GNB3* 825 CC ($n=18$); $^*P<0.05$.

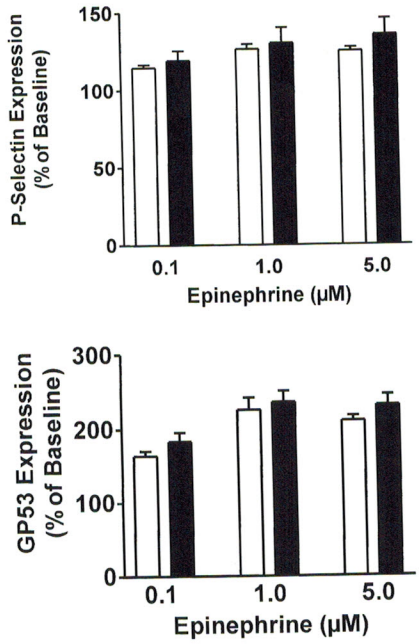

Fig. 5. Expression of P-selectin (CD62) and GP53 (CD63) after epinephrine in the presence of 0.5 μM ADP. ■ *GNB3* 825 TT and TC ($n = 14$); □ *GNB3* 825 CC ($n = 16$).

cluding mainly PTX-sensitive G proteins whereas no interference with signaling via PTX-insensitive G proteins is observed. Exclusively the secondary, irreversible aggregation which comes along with secretion at the plasma membrane [15] is enhanced in platelets of 825T allele carriers in response to epinephrine. Thus, we decided to measure platelet overall secretion by flow cytometric analysis. However, neither the expression of P-selectin (CD62) as a marker of α degranulation, nor that of GP53 (CD63) as a marker of dense granule degranulation displayed differences between the genotypes (Fig. 5). As aggregation in response to platelet secretion products, such as ADP and TXA_2, was similar between genotypes at *GNB3* (Fig. 2), it appears unlikely that differences in platelet secretion can explain the increased responses of 825T allele carriers to epinephrine.

To further investigate the potential mechanisms resulting in the enhanced platelet response to epinephrine in carriers of the 825T allele at *GNB3*, we investigated cAMP levels at baseline and the inhibition of forskolin-stimulated adenylyl cyclase activity by 1 and 10 μM epinephrine. These were, however, not significantly different between genotypes (Fig. 6). Likewise, Pietruck et al. [20] also found no significant differences regarding inhibition of forskolin-stimulated cAMP levels in skin fibroblasts with an overall increased reactivity of PTX-sensitive G proteins which was later shown to depend on the presence or absence of an 825T allele at *GNB3* [9].

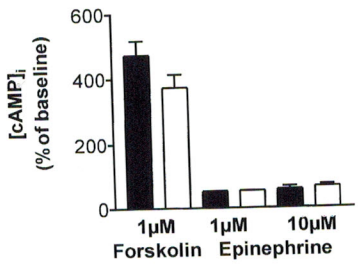

Fig. 6. Intracellular cAMP concentrations after 1 h incubation with 1 μM forskolin and after inhibition of adenylylcyclase by simultaneous incubation with epinephrine. ■ *GNB3* 825 TT and TC ($n = 9$); □ *GNB3* 825 CC ($n = 11$).

Future studies will have to unravel the step(s) which ultimately result in enhanced epinephrine-mediated aggregation in platelets from 825T allele carriers, which appears independent of inhibition of adenylyl cyclase activity or platelet secretion. Since epinephrine is commonly regarded a modulator of platelet activation by weak agonists [16], a function which appears to be also enhanced in our population, our findings lead to the hypothesis that under physiological conditions, e.g. during sympathetic activation, the enhanced reactivity of platelet Gαi proteins in 825T allele carriers may increase platelet aggregation in vivo. Whether or not the observed difference concerning the response to acetylsalicylic acid may also affect the therapeutic use of the substance as anti-aggregatory therapy remains to be determined.

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