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Prothrombinase formation at the site of microvascular injury and aspirin resistance: the effect of simvastatin

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The production of thrombin is determined by the formation of the prothrombinase complex, which assembles predominantly on activated platelets. Aspirin that suppresses the synthesis of thromboxane A₂ (TXA₂) has been shown to reduce thrombin formation at the site of injury [1]. We have reported that impaired platelet sensitivity to aspirin, called aspirin resistance, is associated with faster thrombin generation following injury [2].

Interestingly, statins have been shown to decrease thrombin generation [3] and TXA₂ formation [4,5]. It is unknown whether a statin can inhibit prothrombinase formation to a similar extent in both aspirin-resistant and -sensitive subjects.

We studied 139 consecutive male outpatients at an increased cardiovascular risk, who had low-density lipoprotein cholesterol (LDL) above 3.0 mmol/l and did not take cholesterol-lowering drugs within the 6 weeks preceding the enrollment. Exclusion criteria were: diabetes mellitus; any acute illness; cancer; hepatic or renal dysfunction; therapy with anticoagulants or thienopyridines; documented cardiovascular event; recent ingestion of non-steroidal anti-inflammatory drugs other than aspirin (within one week); history of bleeding or venous thromboembolism. All individuals self-reported the intake of low-dose aspirin as prophylaxis for at least two weeks.

Study participants (n=139) were given simvastatin (Zocor, Merck Sharp and Dohme, 40 mg/d) combined with aspirin at the same dose for 3 months. The University Ethical Committee approved the study, and patients provided written, informed consent. On all individuals 2-4 hours after witnessed aspirin intake, fasting blood samples were obtained prior to and following 12±2 weeks of simvastatin treatment. Aspirin resistance was defined as the occurrence of at least 20% platelet aggregation in platelet-rich plasma (PRP) following stimulation by 1.2 mmol/L arachidonic acid (AA) using a Chrono-Log Lumi-aggregometer (model 490, Chronolog, Haverton, PA). Patients were separated into two groups: aspirin-resistant (n=13) and aspirin-sensitive individuals (n=126). Aspirin (50 µmol/l, 10 min) added to PRP in aspirin-resistant patients reduced platelet aggregation but not normalized it. To assess functional relevance of residual TXA₂, a receptor TXA₂ antagonist, 30 µmol/l 13-azaprostanoic acid (Sigma Aldrich) was added for 3 min to PRP from aspirin-resistant subjects (n=10) on a separate day after the completion of the study (in all subjects, persistent

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>20% aggregation). Serum TXB₂ levels and 8-epi-prostaglandin F_{2α} (8-epi-PGF_{2α}), a marker of lipid peroxidation (both, Cayman Chemicals, Ann Arbor, MI), and plasma prothrombin fragment 1.2 (F1.2), a thrombin generation marker (Dade Behring, Marburg, Germany) were determined.

We assessed prothrombinase formation triggered by vascular injury using a model of microvascular injury in all 13 aspirin-resistant subjects and 13 well matched aspirin-sensitive counterparts selected from the whole group of 126 individuals. Briefly, blood oozing from the bleeding-time wounds performed with a Simplate II device (Organon Teknika) on a forearm was collected every 30 seconds into heparinized capillary tubes (Kabe Labortechnik, Numbrecht-Elsenroth, Germany) and mixed with anticoagulants as described [1]. The molar prothrombinase concentration was calculated assuming that this variable is proportional to the rate of thrombin generation in our model and it was expressed in arbitrary units. The rate of thrombin generation while calculating prothrombinase levels was estimated based on the quantitative immunoblotting of the thrombin B-chain formation. Inter- and intraassay coefficients of variation for the prothrombinase results ranged from 7 to 9 %. Moreover, F1.2 levels were measured in the supernatant samples.

Data are expressed as mean±SD or otherwise stated. Between-group comparisons were done by Student's unpaired *t* test, the Mann-Whitney U test and the chi² test as appropriate. Spearman's rank correlation coefficient was calculated to test the association between 2 variables. A p-level <0.05 was considered significant.

Thirteen aspirin-resistant patients (9.3%) were identified based on AA-induced platelet aggregation (Table). Upon TXA₂ receptor inhibition, baseline aggregation was further impaired in aspirin-resistant individuals (p=0.03), but still higher than in sensitive subjects (13.4±3.1 vs 3.3±2.2 %, p=0.02). Aspirin-resistant subjects were similar to 13 aspirin-sensitive men with regard to age (mean, 55 years), cardiovascular risk factors (5 current smokers [39%], 8 hypertensive subjects [62%] in both groups), platelet count, glucose, creatinine, C-reactive protein, and F1.2. LDL cholesterol was elevated in aspirin-resistant individuals (Table). Serum TXB₂ and plasma 8-epi-PGF_{2α} were higher in aspirin-resistant men, while bleeding time was shorter in this group (Table).

Maximum rates of prothrombinase formation at the site of injury observed between 60 to 120 s was higher in the aspirin-resistant group (Table). Maximum levels of the prothrombinase complex did not differ in relation to responsiveness to aspirin (Table). Maximum rate of F1.2 formation and peak F1.2 levels following injury were markedly higher in aspirin-resistant patients (Table).

After a 3-month simvastatin administration, AA-induced platelet aggregation was more inhibited in aspirin-resistant patients with no change in the aspirin-sensitive group (Table). There was no difference in cholesterol-lowering and anti-inflammatory effects of simvastatin between the groups (Table). In aspirin-resistant and -sensitive subjects, serum TXB₂ and plasma 8-epi-PGF_{2α} levels decreased significantly (Table). Plasma F1.2 remained unchanged. There were no associations between simvastatin-induced reduction in cholesterol and those in TXB₂ and plasma 8-epi-PGF_{2α} levels (data not shown).

After simvastatin, maximum prothrombinase levels and maximum rates of its formation decreased to a similar extent in aspirin-resistant and -sensitive patients (Table). Aspirin resistance, however, was associated with faster posttreatment prothrombinase generation within the first 1 minute with the similar profile in the subsequent minutes compared to values obtained for aspirin-sensitive subjects. Posttreatment maximum rate of F1.2 formation and local peak F1.2 levels were higher in aspirin-resistant than in aspirin-sensitive patients with significant reductions of both parameters following simvastatin (Table). There

was no correlation between bleeding time and the rate of F1.2 or prothrombinase formation or their maximum levels (data not shown).

The present study is the first to show that aspirin resistance is associated with faster prothrombinase formation at the early phase following vascular injury. It might be hypothesized that impaired inhibition of platelet activation, reflected by higher TXA₂ production in aspirin-resistant subjects, leads to more vigorous prothrombinase formation upon vascular injury. Smaller differences in prothrombinase formation following injury observed between both groups before and after statin administration, as compared to those in F1.2 generation, resulted mainly from a faster increase in F1.2 formation than in thrombin B-chain release from prothrombin, a major determinant of prothrombinase formation in our assay [1], in the aspirin-resistant group. This indicates that generation of the prothrombin activation products might be altered by platelet hyporesponsiveness to aspirin.

A small difference in circulating TXB₂ levels, in contrast to a about 20% difference in AA-induced platelet aggregation, was demonstrated to be functionally relevant upon addition of the TXA₂ receptor antagonist, like in the study by Pulcinelli et al [6]. It might be speculated that increased TXB₂ levels in aspirin-resistant patients enhance oxidative stress, reflected by 8-isoprostanes, thus contributing to sustained platelet activation [7] leading to a larger catalytic surface for the prothrombinase formation.

Importantly, we found that simvastatin administration can enhance the antithrombotic properties of aspirin [3] both in aspirin-resistant and-sensitive subjects, which is associated with lower posttreatment TXB₂ levels. These findings extended the observations of Eikelboom et al. [8], who demonstrated that in aspirin-treated high-risk patients, statin treatment is associated with lower urinary 11-dehydro TXB₂ concentrations. Statins have been reported to possess antiplatelet properties in part attributable to their cholesterol-lowering effects [3-5], however this study suggests cholesterol-independent mechanisms of thrombin-lowering effects of simvastatin. Decreased thrombin formation in platelet-rich plasma following pravastatin administration has been convincingly demonstrated by Aoki and coworkers [9]. Using a different methodological approach, Lindhout et al. [10] failed to show the inhibition of the platelet procoagulant response by statins in vitro. Given inconsistent data on platelet-mediated antithrombotic effects of statins in vitro [3], our model of vascular injury that allows to combine the effects of circulating blood cells, vascular wall and plasma components on thrombin formation provides more physiologically relevant data regarding the impact of statins on coagulation triggered by injury as compared to results of in vitro experiments in plasma.

Our findings provide further insights into mechanisms underlying prothrombotic effects of aspirin resistance and reveal an additional modulatory antithrombotic effect of statin administration showing that simvastatin may attenuate the prothrombotic consequences of aspirin resistance in at-risk subjects.

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Table

Effects of simvastatin (40 mg/d) added to aspirin on laboratory variables.

	Aspirin-resistant, n=13			Aspirin-sensitive, n=13		
	before	after	p	before	after	p
TC, mmol/L	7.12±0.51	4.78±0.46	<0.0001	6.02±0.46	4.79±0.46	<0.0001
LDL-C, mmol/L	4.81±0.44	2.75±0.45	<0.0001	3.98±0.36	2.68±0.49	<0.0001
HDL-C, mmol/L	1.35±0.17	1.39±0.25	NS	1.31±0.17	1.39±0.28	NS
TG, mmol/L	2.15±0.45	1.43±0.45	0.003	1.94±0.57	1.50±0.54	0.0004
CRP, mg/L	1.79±0.77	0.98±0.27	0.009	1.89±0.65	0.99±0.39	<0.0001
TXB ₂ , ng/mL	2.3±0.5	0.8±0.3	<0.0001	1.2±0.5	0.9±0.4	<0.0001
8-epi-PGF _{2α} , pg/mL	204.3±39.7	169.4±27.9	0.002	138.1±20.4	121.3±15.1	<0.0001
platelets, 10 ³ /μL	249±38	239±32	NS	246±36	260±35	NS
bleeding time, s	396±15	450±10	0.002	451±10	473±30	NS
AA aggreg, %	23±3.6	14±1.2	0.0009	6.4±2.8	5±1.9	NS
PROTHR max, AU/L	23.9±3.8	15.2±2.4	0.003	22.4±4.1	14.2±1.9	0.001
PROTHR rate, AU/L/s	0.31±0.3	0.19±0.01	0.04	0.22±0.03	0.16±0.02	0.005
F1.2, nmol/L	0.76±0.19	0.73±0.17	NS	0.75±0.18	0.75±0.20	NS
F1.2 max, nmol/L	131.2±23.3	68.8±13.3	<0.0001	97.4±24.6	57.7±10.2	<0.0001
F1.2 rate, nmol/L/s	0.58±0.15	0.33±0.09	<0.0001	0.45±0.13	0.24±0.09	<0.0001

Data are shown as the mean±SD

Abbreviations – TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; CRP, C-reactive protein; 8-epi-PGF_{2α}, 8-epi-prostaglandin F_{2α}; AA aggreg, arachidonic acid-induced platelet aggregation; PROTHR, the estimated prothrombinase concentration using quantitative immunoblotting; F1.2, plasma prothrombin fragment 1.2; max, maximum levels in the bleeding-time blood; rate, maximum rate of the increase in levels in the bleeding-time blood. NS - non-significant. p* denotes comparison between aspirin-resistant versus -sensitive subjects before simvastatin treatment. p** denotes comparison between aspirin-resistant versus -sensitive subjects after simvastatin treatment.