Variations in Platelet Proteins Associated With ST-Elevation Myocardial Infarction

Novel Clues on Pathways Underlying Platelet Activation in Acute Coronary Syndromes

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Objective—Our aim in this study was to provide novel information on the molecular mechanisms playing a major role in the unwanted platelet activation associated with ST-elevation myocardial infarction (STEMI).

Methods and Results—We compared the platelet proteome of 11 STEMI patients to a matched control group of 15 stable chronic ischemic cardiopathy patients. In addition, we did a prospective study to follow the STEMI patients over time. Proteins were separated by high-resolution 2D gel electrophoresis, identified by mass spectrometry, and validated by Western blotting. Platelets from STEMI patients on admission displayed 56 protein spot differences (corresponding to 42 unique genes) compared with the control group. The number of differences decreased with time during the patients' follow-up. Interestingly, the adapter protein CrkL and the active form of Src (phosphorylated in Tyr418) were found to be upregulated in platelets from STEMI patients. Major signaling pathways related to the proteins identified include integrin, integrin-linked kinase, and glycoprotein VI (GPVI) signaling. Interestingly, a study on an independent cohort of patients showed a higher degree of activation of GPVI signaling in STEMI patients.

Conclusion—CrkL, the active form of Src, and GPVI signaling are upregulated in platelets from STEMI patients. **(*Arterioscler Thromb Vasc Biol*. 2011;31:2957-2964.)**

Key Words: acute coronary syndromes ■ platelets ■ GPVI signaling ■ proteomics

Acute coronary syndromes (ACSs) encompass unstable angina, non-ST segment elevation (NSTE) myocardial infarction, and ST-segment elevation myocardial infarction (STEMI). The latter usually occurs when thrombus forms on a ruptured atheromatous plaque and causes a prolonged occlusion of an epicardial coronary artery.[1] There is no doubt platelets play a fundamental role in the pathogenesis of an ACS, being implicated in the thrombus formation that follows the atheroma plaque rupture.[2] Activated platelets release proteins in the coronary circulation of ACS patients, such as matrix metalloproteinases, that contribute to sustained intracoronary platelet activation.[3] Following an ACS, antiplatelet therapy is recommended to reduce the growth of a thrombus. Primary targets of such therapy are molecules involved in platelet activation and aggregation. Nevertheless, there is a need for the development of a new generation of safer and more effective antithrombotic drugs with larger therapeutic windows and for a better understanding of the pathogenic processes that lead to thrombotic occlusion of blood vessels linked to an acute event.[2]

Proteomics has emerged as a promising tool in cardiovascular research, and more precisely in the study of ACSs.[4] Studies have included plasma, monocytes, and, very recently, platelets.[5–8] The objective of these studies was the identification of novel screening and diagnostic biomarkers that might help to predict the disease and provide novel information on the molecular events associated with it, hopefully leading to the identification of novel drug targets. We have been involved in the platelet studies and thus have recently published a high-resolution 2D gel electrophoresis–based study of the platelet proteome of patients with NSTE-ACS.[7] We now present the first analysis of the platelet proteome from patients with STEMI compared with matched stable coronary artery disease (SCAD) controls. We also did a prospective study to follow the STEMI patients over time and a specific analysis of the collagen receptor glycoprotein VI (GPVI) signaling cascade in an independent cohort of patients. Our primary objective was to provide, through a global proteomic approach, novel information on the molecular mechanisms playing a major role in the unwanted platelet activation associated with STEMI.

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Methods

Patients

Eleven patients admitted into a tertiary hospital in the northwest of Spain presenting with STEMI (defined as angina pain of at least 20 minutes' duration with elevated cardiac enzymes and ST-segment elevation of at least 0.1 mV in 2 or more contiguous leads or presumably new left bundle branch block) entered the study. Exclusion criteria were inflammatory or neoplastic diseases; coagulation disorders; platelet-associated disorders; other significant heart disease except left ventricular hypertrophy secondary to hypertension; chronic drug therapy (except for drugs required to treat preexisting clinical atherosclerosis or its risk factors); and having experienced surgical procedures, major traumatisms, thromboembolic events, or revascularization procedures in the previous 3 months.

The study was approved by the local Ethics Committee (Galician Clinical Investigation Ethics Committee) and developed according to the principles outlined in the Declaration of Helsinki. At the moment of diagnosis, the patients were asked to participate in the study. In case of acceptance, they signed the informed consent, and 27 mL of blood were collected in sodium citrate BD Vacutainer tubes for analysis. All samples were obtained within the first 12 hours following the initiation of the symptoms, after arrival at the emergency department. In most cases, patients were administered aspirin before arrival to hospital, so aspirin-pretreated patients were admitted in the study. Some patients had also clopidogrel treatment and were also admitted. However, patients previously treated with anti-$\alpha_{IIb}\beta_3$ drugs were excluded because in this case discrimination was easier (these drugs are administered at the hospital). When anti-$\alpha_{IIb}\beta_3$ drugs had been already administrated before blood extraction, we decided to exclude those patients to minimize the use of antiplatelet drugs in the study and also because it would have been difficult to find good SCAD matched controls. A second blood sample was taken on day 5 to investigate whether there was a fast reversal of the changes observed in the platelet proteome on admission. Another blood sample was taken after 6 months. Blood was also collected from 15 matched SCAD patients experiencing stable chronic ischemic cardiopathy. Such a group was decided to be the most adequate control group because of the clinical characteristics of the STEMI patients. SCAD patients were required not to have a history of acute cardiovascular event within the year before the inclusion in the study. An age- and sex-matched healthy control group consisting of 10 volunteers was also included for validation studies.

Platelet Isolation

Platelets were isolated by an established method that limits contamination from other blood cells as described previously.[9] Washed platelets were resuspended in Tyrodes-HEPES (134 mmol/L NaCl, 0.34 mmol/L Na_2HPO_4, 2.9 mmol/L KCl, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, 5 mmol/L glucose, 1 mmol/L $MgCl_2$, pH 7.3) at 6×10^8 platelets/mL and allowed to rest for 30 minutes at room temperature. Platelets were then spun down at 10 000 *g* in the presence of 1 mmol/L EGTA and, after addition of 5 μL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), immediately frozen in liquid nitrogen for a few seconds followed by the addition of 2D gel electrophoresis sample buffer (see below). Protein samples were stored at −80°C.

For GPVI signaling studies, 500-μL aliquots of platelets (6×10^8/mL) were warmed at 37°C for 10 minutes in the presence of 1 mmol/L EGTA, 10 μmol/L indomethacin, and 2 U/mL apyrase before stimulation for 90 seconds with collagen-related peptide (CRP) (final concentration of 10 μg/mL). Stimulations were with constant stirring at 1200 rpm in a Chrono-log aggregometer. Unstimulated (basal) samples were processed in parallel but treated with CRP diluent (0.01 mol/L acetic acid containing 0.1% fatty acid–free BSA) alone. Basal and stimulated platelets were lysed for Western blot analysis as described previously.[10]

Proteomic Analysis

Protein quantitation was done with the Coomassie Plus protein reagent (Thermo Scientific, Asheville, NC). Six hundred micrograms of protein were loaded onto each gel to allow detection of low abundant proteins. An individual gel was run for each blood sample, from each STEMI patient, at 3 different times: on admission, after 5 days, and after 6 months. One sample was obtained from each SCAD patient. The remaining protein was used for Western blotting.

For each sample, protein was dissolved in 500 μL of 2D sample buffer (5 mol/L urea, 2 mol/L thiourea, 2 mmol/L tributylphosphine, 65 mmol/L dithiothreitol, 65 mmol/L CHAPS, 0.15 mol/L NDSB-256, 1 mmol/L sodium vanadate, 0.1 mmol/L sodium fluoride, and 1 mmol/L benzamidine). Ampholytes (Servalyte 4-7) were added to the sample to a final concentration of 1.6% (v/v). The first dimension was on immobilized pH gradient strips 4 to 7, 24 cm (GE Healthcare). The second dimension was by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gels. Protein staining was with Sypro Ruby fluorescent dye. Differential image analysis was with Ludesi REDFIN 3 software (Lund, Sweden). Further information on the 2D gel electrophoresis protocol and image analysis can be found in the Supplemental Methods, available online at http://atvb.ahajournals.org.

Protein features chosen for mass spectrometric analyses were excised from the gels and in-gel digested with trypsin. Proteins were identified using a 4800 matrix-assisted laser desorption ionization/tandem time of flight analyzer (Applied Biosystems) and a Bruker Amazon electron transfer dissociation ion trap. A database search was performed with the Mascot v2.1 search tool (Matrix Science, London, United Kingdom) screening SwissProt (release 56.0). Further details on the analysis can be found in the Supplemental Methods.

Western Blotting

Western blot analyses were carried out on polyvinylidene difluoride membranes using the following primary antibodies and dilutions: rabbit anti-CrkL (1:500) from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), rabbit anti-Src (pTyr418) (1:1000) from Invitrogen (Camarillo, CA), mouse anti-phosphotyrosine monoclonal antibody (clone 4G10) (1:1000) from Millipore (Billerica, MA), and mouse anti-GAPDH (0.5 μg/mL) from Ambion (Austin, TX). Membranes were exposed to horseradish peroxidase–labeled goat anti-rabbit or goat anti-mouse antibodies (dilution 1:5000) (Pierce, Rockford, IL), processed using an enhanced chemiluminescence system (ECL, Pierce), and quantified by densitometry. Further details on the Western blot protocol can be found in the Supplemental Methods.

Ingenuity Pathways Analysis

Ingenuity Pathways Analysis software (Ingenuity Systems) was used to investigate possible interactions between all the identified proteins. Interactive pathways were generated to observe potential direct and indirect relations among the differentially expressed proteins.

Statistical Analysis

Data for categorical or dichotomous variables are expressed as percentages and were compared using the χ^2 test or the Fisher exact test. Unless stated otherwise, data for continuous variables are expressed as the median and interquartile range and were compared by Mann-Whitney test. The differential proteomic analysis was done analyzing all the spots between patients with STEMI and SCAD; for a given spot, the probability value was calculated using the quantified and normalized volumes for the matched spot in each of the images and applying the Mann-Whitney test.

All probability values were 2-tailed, and values of <0.05 were considered to indicate statistical significance. All analyses were performed using SPSS 17.0 software for Windows (SPSS Inc, Tokyo, Japan).

Results

Patients' Characteristics

Eleven patients admitted to hospital with the diagnosis of STEMI and 15 age and gender-matched patients with SCAD

Table 1. Clinical Characteristics of ST-Elevation Myocardial Infarction and Stable Coronary Artery Disease Patients (Proteomic Study)

Variable	Acute Myocardial Infarction (n=11 Patients)	Stable Coronary Artery Disease (n=15 Patients)
Age, y	69 (50–79)	67 (47–73)
Females, %	27.3	20.0
Hx arterial hypertension, %	45.5	60.0
Hx diabetes mellitus, %	18.2	26.7
Hx dyslipidemia, %	36.4	40.0
Hx coronary artery disease, %*	18.2	100.0
Platelets/μL	268 000 (178 000–303 000)	223000 (199 000–239 500)
Treatments		
Aspirin, %	90.9	100.0
Clopidogrel, %	36.4	40.0
Other antiplatelets, %	0	0
Anticoagulants, %	0	0

Data are presented as the median and interquartile range or percentage of patients. Further information can be found in Supplemental Table I. Hx indicates medical history.

*$P<0.05$.

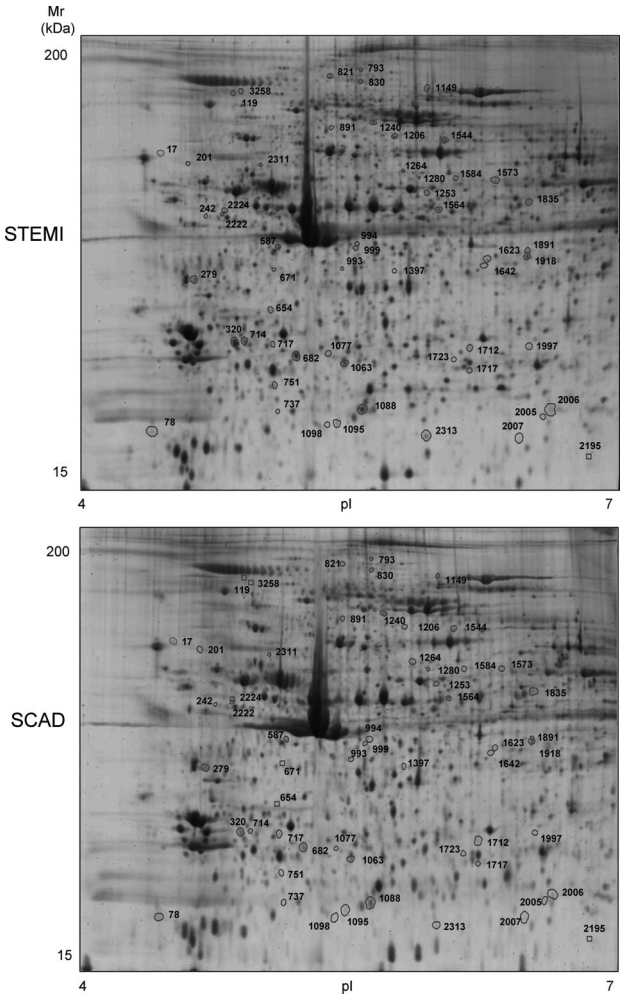

Figure 1. High-resolution 2D gel electrophoresis–based proteome analysis of platelets from ST-segment elevation myocardial infarction (STEMI) patients. Shown are representative 2D gel electrophoresis gel images of STEMI and stable coronary artery disease (SCAD) circulating platelets (isoelectric focusing: pH range 4–7; second dimension: 10% SDS-PAGE). The figure shows the location on the 2D gels of those spots differentially regulated when comparing STEMI patients and SCAD controls. Protein identifications are shown by the identification numbers in Supplemental Table II. Further information can be found in Supplemental Table III.

were included in the proteomic study. The matching was also based on antiplatelet treatment performed before blood extraction. There were only a few clinical differences between both groups, as shown in Table 1 and Supplemental Table I. Patients included in the SCAD control group had a higher prevalence of 1-vessel coronary disease, whereas in STEMI patients, complex, multivessel lesions were more frequent. Prehospital admission use of angiotensin-converting enzyme inhibitors and statins was more prevalent in the SCAD group. Finally, regarding laboratory results, patients with acute events presented higher leukocyte and glucose levels and lower protein and mean platelet volume compared with stable patients.

Platelet Proteome Analysis of STEMI Patients

A mean ($\pm$SD) of 2426$\pm$53 protein features were found on pI 4 to 7 gels corresponding to platelets from SCAD patients, whereas 2466$\pm$56 protein features were found in the STEMI samples. We focused on the identification of disappearing and appearing spots, as well as up- and downregulation of spot intensities where the fold change was at least 1.5 (with $P<0.05$). By applying these criteria, 56 protein features were detected as differentially regulated when comparing the platelet proteome of STEMI patients on admission versus SCAD controls (Figure 1). All protein features were successfully identified by mass spectrometry. They corresponded to 42 different open reading frames (Supplemental Tables II and III). Forty of the protein features identified were upregulated in STEMI samples gels, whereas 16 were downregulated. Eight proteins were represented in the gels by more than 1 spot, and 8 spots had more than 1 protein, normally from the same family.

The number of proteins differentially regulated in platelets from STEMI patients, as compared with stable controls, was decreasing with time. As mentioned above, on admission there were 56 differentially regulated protein features. Of those 56, only 32 remained significantly different at day 5. After 6 months, only 4 protein spots remained significantly different (Supplemental Table IV).

The 42 differentially regulated proteins identified in the STEMI study can be divided in the following functional groups: cytoskeletal (38%), signaling (24%), extracellular/vesicles/secretory trafficking pathway (21%), and other (17%). A close-up view of a selection of differentially regulated proteins is shown in Figure 2.

A selection of proteins was validated by Western blotting. Below, we present data on signaling proteins.

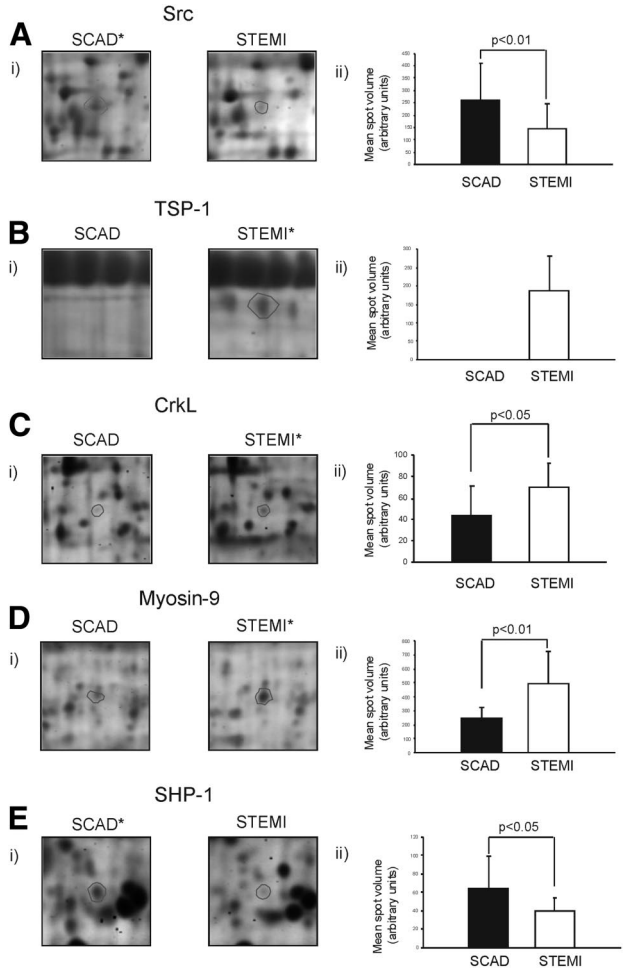

Figure 2. Selection of proteins differentially regulated between ST-segment elevation myocardial infarction (STEMI) and stable coronary artery disease (SCAD) patients. Enlargement of representative spots for Src (spot no. 1835) (**A**), thrombospondin-1 (spot no. 119) (**B**), CrkL (spot no. 1642) (**C**), myosin-9 (spot no. 1918) (**D**), and SH2 domain-containing tyrosine phosphatase-1 (spot no. 1723) (**E**). Left (i), representative 2D gel electrophoresis images. Right (ii), bar graph for each spot showing variations in mean volume±SD in different groups: SCAD (n=15), and STEMI (n=11). *Group of patients with higher expression of the indicated protein.

Active Src (pTyr418) Is Upregulated in Platelets From STEMI Patients

Our proteomic analysis revealed 1 spot corresponding to Src downregulated in platelets from STEMI patients (Figure 2A). Src is a 60-kDa nonreceptor tyrosine kinase involved in initiating signaling from various tyrosine kinase-linked platelet receptors.[11] Src and other members of the Src kinase family are tightly regulated by tyrosine phosphorylation, so we hypothesized that the difference we were observing could be due to Src phosphorylation/dephosphorylation. Full catalytic activity of Src requires phosphorylation of tyrosine 418, which is located in the catalytic domain. By using a specific anti-Src (pTyr418) antibody, we demonstrated that the active form of Src was upregulated in platelets from STEMI patients (Figure 3). This apparent contradiction with the proteomic results is not actually a contradiction: when there is an increase in phosphorylation of a given protein, spots corresponding to hypophosphorylated forms may diminish in intensity, whereas the intensity of spots corresponding to hyperphosphorylated forms, in a more acidic region, goes up. In our case, we did not detect the latter. Some possible reasons for that are that spots of interest were hidden by other surrounding spots in the stained gel (corresponding to more abundant proteins) or they were detected but the difference was below the 1.5-fold change cutoff level. We should also bear in mind that Western blotting is much more sensitive than gel staining.

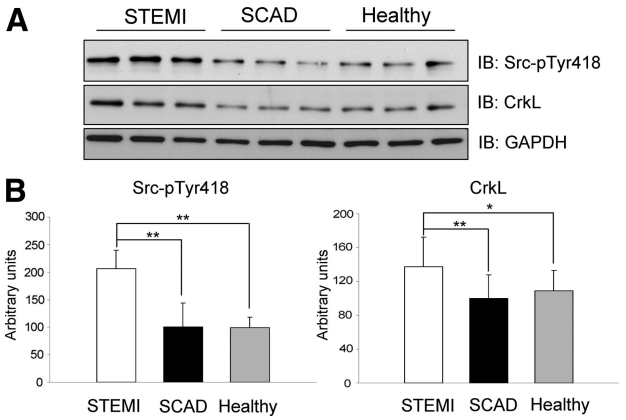

Figure 3. The active form of Src (pTyr418) and CrkL are upregulated in platelets from ST-segment elevation myocardial infarction (STEMI) patients. **A,** Western blot analysis of Src-pTyr418, CrkL, and GAPDH protein expression levels in platelets from STEMI patients and stable coronary artery disease (SCAD) and healthy controls. Images are representative of the results obtained. **B,** Densitometry graph representing the mean values±SD of band intensities corresponding to Src-pTyr418 and CrkL expression assessed by Western blot, with respect to GAPDH, for all the patients included in the study. Statistically significant differences are highlighted. $^{**}P<0.01$; $^{*}P<0.05$. IB indicates immunoblot.

The Adapter Protein CrkL Is Upregulated in Platelets From STEMI Patients

CrkL is a GPVI signaling protein that serves as an adapter for Syk tyrosine kinase.[12] We found this protein in 1 spot upregulated in platelets from STEMI patients (see Figure 2C). Validation studies confirmed this result (Figure 3).

Ingenuity Pathways Analysis

Ingenuity Pathways Analysis software (Ingenuity Systems) was used to investigate possible interactions between all the identified proteins to highlight predominant networks and pathways. Interestingly, 35 of the 42 differentially regulated proteins identified are interconnected as a part of a common network related to tissue and cell morphology (Supplemental Figure I). The top molecular and cellular functions are cell-to-cell signaling and interaction, cellular assembly and organization, and cell morphology (Supplemental Figure IIA). The top 3 canonical pathways related to the proteins identified are actin cytoskeleton signaling, integrin signaling, and integrin-linked kinase signaling (Supplemental Figure IIB). In addition, 7 differentially regulated proteins are also known to be involved in platelet activation by the collagen receptor GPVI: α-actinin-1, Src, SKAP-55 homologue, Gads,[13] SH2 domain-containing tyrosine phosphatase-1,[14]

Table 2. Selection of Proteins Involved in Platelet Signaling Differentially Regulated in ST-Elevation Myocardial Infarction vs Stable Coronary Artery Disease Patients

Protein	Uniprot Code	Spot	Fold Change
14-3-3 protein ζ/δ	1433Z_HUMAN	242	−1.80*
α-Actinin-1	ACTN1_HUMAN	2222	+2.93*
	ACTN1_HUMAN	2224	+2.58*
	ACTN1_HUMAN	2311	+2.32*
Cdc42	CDC42_HUMAN	2006	+1.82*
CrkL	CRKL_HUMAN	1642	+1.63
GADS	GRAP2_HUMAN	2005	−1.79
Inositol monophosphatase 1	IMPA1_HUMAN	717	−1.61
Myosin-9	MYH9_HUMAN	1623	+1.69*
	MYH9_HUMAN	1918	+1.98*
	MYH9_HUMAN	2313	+2.69*
Src	SRC_HUMAN	1835	−1.83
SKAP-HOM	SKAP2_HUMAN	279	−1.51*
Talin-1	TLN1_HUMAN	793	+2.08*
		821	+2.45*
		830	+2.92*
		891	−1.75
		1149	+2.01*
		1206	+3.61*
		1240	−1.71
		1544	+2.28*
		1573	+1.87*
SHP-1	PTN6_HUMAN	1723	−1.61

A negative fold change indicates that the protein feature is downregulated in ST-elevation myocardial infarction, whereas a positive fold change indicates that the spot is upregulated in the acute group. SKAP-HOM indicates SKAP-55 homologue.

*Differentially regulated features have a P value of <0.05 except those marked with an asterisk (*), which have a P value of <0.01.

Cdc42,[15] and CrkL.[12] A selection of differentially regulated proteins related to platelet signaling is shown in Table 2.

GPVI Signaling Is Upregulated in STEMI Patients

As mentioned above, our data suggest the involvement of GPVI signaling in the acute event. To further explore this issue mechanistically, we recruited an independent cohort of patients to investigate the activation state of the GPVI signaling cascade. The clinical characteristics of the patients (Supplemental Table V) were similar to those of the original study. Platelets from 6 STEMI patients, 6 matched SCAD controls, and 6 sex- and age-matched healthy controls were in vitro activated with the GPVI specific agonist CRP in the presence of EGTA (which prevents platelet aggregation during GPVI activation) and inhibitors of secondary mediators (ADP, thromboxanes). Those inhibitors were used to make sure changes in the proteome were specifically due to GPVI signaling.[13] A comparison between basal (unactivated) and CRP-activated platelets was carried out for the 3 groups of patients (STEMI, SCAD, healthy). To do so, equal amounts of protein from each patient belonging to the same group were pooled for each study point (basal, CRP) and loaded in a 4% to 12% gradient Bis-Tris gel. Because GPVI signaling goes primarily through tyrosine phosphorylation, a Western blot was carried out with the 4G10 antiphosphotyrosine antibody. As expected, there was an increase in tyrosine phosphorylation when platelets were activated with CRP, but interestingly, such increase was higher in STEMI patients (Figure 4). Moreover, because Src family kinases play a fundamental role in GPVI signaling, we reprobed the membranes with the anti-Src (pTyr418) antibody, which recognizes the active form of the kinase. As can be seen in Figure 4, CRP stimulation induced an ≈2-fold augment in Src-pTyr418 levels. Interestingly, there was also an increase in Src-pTyr418 levels in platelets from STEMI patients compared with controls, both in basal and CRP-activated platelets. This confirms the data from our proteomic study and shows a higher activation of GPVI signaling in platelets from STEMI patients. Further research is under way to study in detail by proteomics the GPVI signaling cascade in a bigger group of patients to identify those proteins that contribute more importantly to the differences observed.

Discussion

The present study constitutes the first proteomic analysis of platelets from STEMI patients, and it complements our recent study on NSTE-ACS patients.[7] The 2 analyses were done separately because of the clinical characteristics of each group of patients, so the SCAD control groups were different. The results presented here provide novel information on the molecular events related to the unwanted platelet activation that takes place in STEMI, identifying the main pathways involved, and confirming a higher activation of the GPVI signaling cascade in platelets from STEMI patients.

The study design is in agreement with recent platelet proteomic and transcriptomic studies on ACS.[7,16,17] We selected patients with SCAD as a control group to focus on differences linked to atherothrombotic events, bypassing the influence of antiplatelet drugs. Nevertheless, a healthy control group was included during the validation stage, as done in the transcriptomic studies mentioned above. No significant differences were observed between SCAD and healthy groups in the validation studies. Study limitations are mentioned in the discussion section shown in the supplemental material.

Regarding the clinical characteristics of the patients, there was an obvious dissimilarity in treatment of the two study groups, with SCAD patients having more frequent preadmission prescription of angiotensin-converting enzyme inhibitors and statins because this group presented an already established diagnosis of ischemic heart disease, compared with patients with acute events, in whom this diagnosis was mostly performed for the first time. Additional discussion of laboratory differences between patient groups can be found in the supplemental material.

Focusing on the proteomic analysis, we found 56 protein features to be differentially regulated between platelets from STEMI and SCAD patients. The fact that those spots were related to 42 unique genes suggests some cases of extensive posttranslational modifications, quite common in platelets

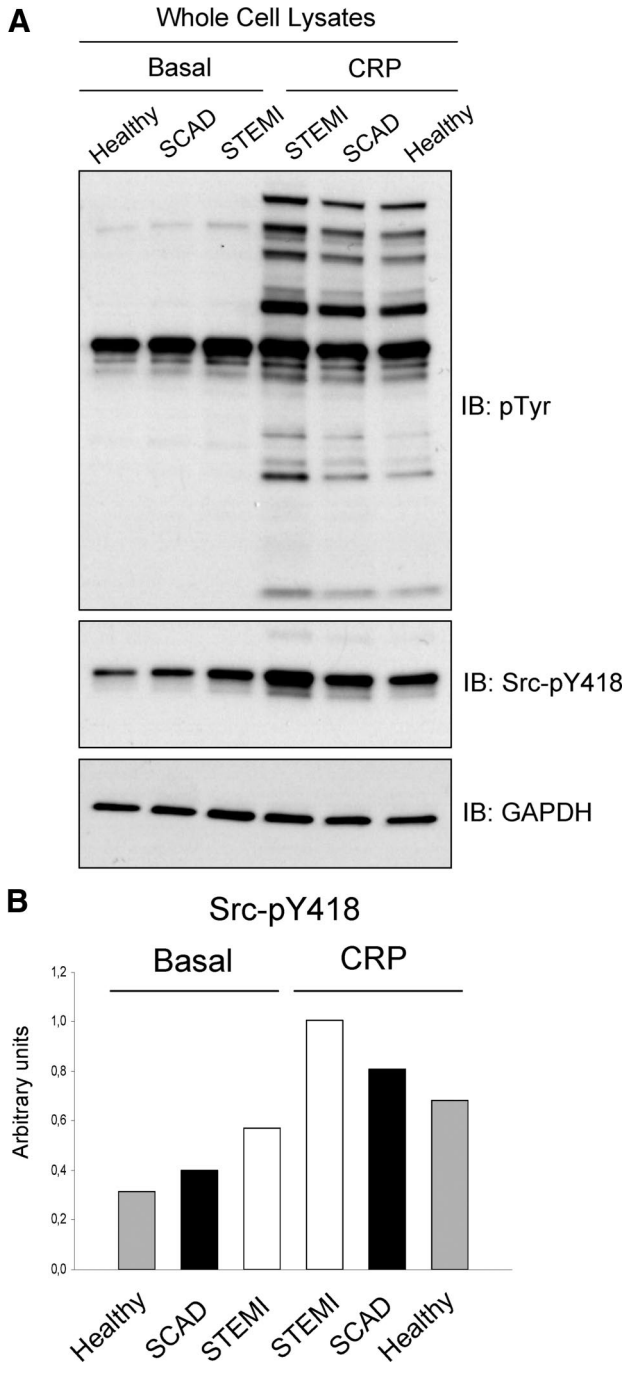

Figure 4. GPVI signaling is upregulated in platelets from ST-segment elevation myocardial infarction (STEMI) patients. **A,** Western blot analysis of pTyr (4G10 monoclonal antibody), Src-pTyr418, and GAPDH protein expression levels in basal and collagen-related peptide (CRP)-activated platelets from STEMI patients (n=6) and stable coronary artery disease (SCAD) (n=6) and healthy (n=6) controls. Equal amounts of protein corresponding to each of the 6 patients per group were pooled for each study point and loaded in different lanes in a 4% to 12% NuPAGE Bis-Tris gel for protein separation before Western blotting. **B,** Densitometry graph representing the values of band intensities corresponding to Src-pTyr418 expression assessed by Western blot, with respect to GAPDH. This analysis corresponds to the blot images shown in **A**. IB indicates immunoblot.

following activation, such as phosphorylations or natural proteolysis. The latter also explains some cases where proteins had an experimental molecular weight significantly lower than the theoretical one. The number of differences was higher than in the NSTE-ACS study we recently carried out—40 protein spots related to 22 unique genes—which is not surprising because the acute event was more severe in the case of STEMI, and blood samples were collected closer to the initiation of the event. That would be also one of the explanations for why there was not a bigger overlap between both studies. Nevertheless, there are similarities, and thus, signaling and cytoskeletal proteins were the main functional groups of proteins identified in both studies, with an important number of differentially regulated proteins involved in integrin and GPVI signaling. Moreover, Ingenuity Pathways Analysis software revealed that most proteins identified in both NSTE-ACS and STEMI studies are involved in a network related to cell morphology.

Signaling Pathways as Possible Future Targets for Antiplatelet Therapy: Upregulation of GPVI Signaling in STEMI

In the past few years, studies identifying platelet receptors and signaling mechanisms have yielded a trove of new targets for antiplatelet therapy.[2,11] For example, recent studies have shown that several cell-surface receptor-ligand interactions occur on close contact between platelets, such as the binding of the ligand semaphorin 4D to its receptors, CD72 and plexin B1. These receptors mediate platelet-platelet interactions and thrombus retraction and hence are attractive therapeutic targets.[18] Proteomics has contributed to depicting the main signaling pathways in platelets, leading to the identification of novel receptors and signaling proteins, some of them potential antithrombotic targets. We have been among the pioneers in the field.[10,13,19,20] In this way, the complexity of outside-in and inside-out signaling has begun to be unraveled. However, redundancy in signaling pathways makes it difficult to identify clear therapeutic targets. Nevertheless, there are some exceptions. For instance, the binding of the cytoskeletal protein talin-1 to the cytoplasmic domain of β_3-integrin was shown to be required for activation of the $\alpha_{IIb}\beta_3$ integrin.[21] Moreover, changing a single amino acid in the cytoplasmic domain of β_3-integrin selectively disrupted talin-1 binding and reduced arterial thrombosis in an animal model[22] showing that blockade of this interaction could be a new antithrombotic strategy. Interestingly, most of the proteins identified in the present study are interconnected in a common network that primarily involves cytoskeletal and signaling proteins, processes closely related to platelet activation. Indeed, integrin and GPVI signaling are among those pathways highlighted by our study. Src family kinases play a major role in these signaling cascades, especially in the case of the collagen receptors GPVI and $\alpha_2\beta_1$ and the fibrinogen receptor $\alpha_{IIb}\beta_3$.[11] Interestingly, we demonstrate the active form of Src is upregulated in STEMI patients, which suggests a primary role for those pathways—where Src family kinases are essential—in the acute event.

The integrin $\alpha_{IIb}\beta_3$, mainly responsible for platelet aggregation, is a well-known antiplatelet target.[23] Integrin $\alpha_2\beta_1$

was the first platelet collagen receptor to be identified and serves as an adhesion receptor,[11] so its involvement in the acute activation of platelets makes sense in a collagen-rich environment. The novelty of our study is to highlight other 3 platelet signaling pathways related to platelet activation in ACS: actin cytoskeleton signaling, integrin-linked kinase signaling, and GPVI signaling. The latter is an interesting target. It has been recently shown that platelet GPVI binds to collagenous structures in the core region of human atheromatous plaque and is critical for atheroprogression in vivo.[24] Indeed, inhibition of GPVI both via GPVI-Fc and anti-GPVI antibodies resulted in protection against atherosclerosis in rabbit and mice models.[25] However, at present there are no drugs in clinical use that block the binding of platelets to collagen and von Willebrand factor and hence their adhesion to the blood vessel wall. Our results reinforce the idea that GPVI signaling, together with integrin and integrin-linked kinase signaling, may be a good antiplatelet target for ACSs. For example, in addition to the Src results, we found CrkL to be upregulated in patients with STEMI. This adapter protein has been shown to be involved in GPVI signaling.[12] CrkL consists of Src homology 2 and Src homology 3 protein-binding domains and mediates assembly of protein complexes in signaling.[26] More precisely, it is an adapter for Syk, a tyrosine kinase critical for collagen induced-platelet activation through GPVI.[27] This association, demonstrated in vivo and in vitro, could provide an explanation for the translocation of Syk to the cytoskeleton during platelet aggregation.[27] As was the case with the active form of Src, the upregulation of CrkL in patients with STEMI could be indicative of an upregulation of pathways where this protein has been reported to play a role, such as the GPVI signaling cascade. We confirmed the latter by a specific GPVI signaling study on an independent cohort of patients. As shown in Figure 4, there was an upregulation of GPVI signaling in STEMI patients. These results confirm our initial data and are also consistent with recent reports that demonstrate that GPVI surface expression is elevated in platelets from ACS patients.[28,29]

Conclusions

This study provides novel information on platelet proteome changes related to STEMI and, in combination with our recent study of platelets from NSTE-ACS patients, allows highlighting of those signaling pathways more implicated in the unwanted platelet activation associated with the acute event: actin cytoskeleton signaling, integrin signaling, integrin-linked kinase signaling, and GPVI signaling. In line with the above, the adapter protein CrkL—known to participate in GPVI signaling—and the active form of the tyrosine kinase Src were found to be upregulated in platelets from STEMI patients. Moreover, we show for the first time an upregulation of GPVI signaling in STEMI patients. We hope that our study will pave the way for future research on novel therapeutic targets and platelet-related biomarkers in ACS.

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Disclosures

None.

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