Protein Kinase C ε Expression in Platelets from Patients with Acute Myocardial Infarction

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Abstract

Objective: Platelets play crucial roles in the pathophysiology of thrombosis and myocardial infarction. Protein kinase C ε (PKCε) is virtually absent in human platelets and its expression is precisely regulated during human megakaryocytic differentiation. On the basis of what is known on the role of platelet PKCε in other species, we hypothesized that platelets from myocardial infarction patients might ectopically express PKCε with a pathophysiological role in the disease.

Methods and Results: We therefore studied platelet PKCε expression from 24 patients with myocardial infarction, 24 patients with stable coronary artery disease and 24 healthy subjects. Indeed, platelets from myocardial infarction patients expressed PKCε with a significant frequency as compared to both stable coronary artery disease and healthy subjects. PKCε returned negative during patient follow-up. The forced expression of PKCε in normal donor platelets significantly increased their response to adenosine diphosphate-induced activation and adhesion to subendothelial collagen.

Conclusions: Our data suggest that platelet generations produced before the acute event retain PKCε-mRNA that is not down-regulated during terminal megakaryocyte differentiation. Results are discussed in the perspective of peri-infarktual megakaryocytopenesis as a critical component of myocardial infarction pathophysiology.
Platelet PKCε in Myocardial Infarction

Platelet activation

Aliquots of whole blood samples were stained with anti-CD62p monoclonal antibody (mAb) as a marker of platelet activation and α-granule release and analyzed by flow cytometry [38]. Briefly, 1:100 PBS-diluted whole blood was incubated with the mAb CD62p-FITC (anti P-selectin, Pharmingen Becton Dickinson, San Diego, CA) in the presence of incremental doses of ADP (0; 1,25; 2,5; 5 μM). After 20 min at room temperature, 400 μl of 2% buffered paraformaldehyde was added for fixation.

Analysis of the samples was performed by an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA) and the Expo ADC software (Beckman Coulter). In some experiments, the absolute number of surface antigens expressed/cell was calculated. To this purpose, the flow cytometer was calibrated with a set of standardized beads (DAKO, Glostrup, Denmark) each with a known amount of fluorochrome (either FITC or PE) expressed in units of MESF (Molecules of Equivalent Soluble Fluorescein). Thus, a standard curve was constructed by plotting MESF values for the beads against the median channel in which the peak was displayed.

Platelet purification

All the remaining blood samples were centrifuged at 160 g for 20 minutes at room temperature (RT), to obtain platelet rich plasma (PRP). Platelets were then purified by negative separation using magnetic beads coated with anti-CD45 antibodies (Dynabeads®, Invitrogen, Carlsbad, CA), to deplete nucleated cells. Briefly, PRP were stained with the magnetic beads-coated mAb anti-CD45 for 20 min at RT on a rotator. Purified platelets were washed 3 times in PBS/BSA solution, counted, tested for purity by anti-CD41 staining and flow cytometry analysis (only samples where CD41+ cells >90% were used), and finally processed for RNA extraction.

RNA isolation

Highly purified platelets were treated with an appropriate amount of TRizol® (Invitrogen) for cell lysis and RNA extraction, following the manufacturer’s protocol. Briefly, chloroform was added to TRizol®-treated samples and centrifuged at 12,000 g for 15 min at 4°C. The acqueous phase, containing RNA, was transferred in a new tube and added with an equal volume of isopropanol. After incubation, the samples were centrifuged at 12,000 g for 15 min at 4°C, to obtain RNA pellets that were washed and resuspended in DEPC-treated water for quantification by spectrophotometer.

Amplification of RNA for PKCε gene expression analysis

The isolated RNA was both positively and negatively tested for cell population purity by Reverse-Transcription PCR (RT-PCR). A standard set of primers was used to test platelet RNA (amplification of CD41) or contaminant cells RNA (amplification of CD45 for nucleated cells).

Platelet RNA purification was followed by reverse transcription and RT-PCR to yield complementary DNA (cDNA). From the cDNA sample, cRNA was synthesized by in vitro transcription (IVT), and then analyzed for PKCε gene expression. Briefly, 1 μg total RNA was reverse transcribed with MMV reverse transcriptase and subjected to PCR amplification to detect CD41, CD45 and PKCε cDNA.

PCR were performed under the following reaction conditions: 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. We used 35 cycles of amplification. The sequences of primers used for PCR were: CD41: 5’-GGAAT

Methods

Patients

Three groups of subjects were studied: 1) twenty-four acute myocardial infarction patients (MI) with an ST-segment elevation; 2) twenty-four patients with newly-diagnosed stable CAD (sCAD) and 3) twenty-four healthy subjects (HD). Patients were enrolled at the Cardiology Division of the Azienda Ospedaliero-Universitaria of Parma after written informed consent was obtained and the study was performed according to the Declaration of Helsinki. The protocol was approved by the unique Local Ethical Committee of the Ospedale Maggiore of Parma and University of Parma. Blood collection from MI patients was accomplished within 12 hours from the acute event (in most cases within 3–4 hours) and before any invasive procedure or pharmacological treatment was performed. Previous antiplatelet therapy was an exclusion criteria in all groups. Patients characteristics and their cardiovascular risk factors are reported in Table 1.

Fifty ml of citrate anti-coagulated blood samples were taken from patients and controls (collected in Vacutainers, 3.8% sodium citrate final concentration; BD Vacutainer, Becton Dickinson, San Diego, CA) for subsequent analyses.
GTGCA GGGCT TTGAG-3’ (sense) and 5’-GGCTG TTCTT GCTCC GTATC-3’ (antisense); CD45: 5’-GGAAG TGCTG CAATG TGTCA TT-3’ (sense) and 5’-CTTGA CATGC ATACT ATTAT CTGAT GTCA-3’ (antisense); PKCe: 5’-CACCA TCCAG TTTGA GGAGC-3’ (sense) and 5’-CGACC CTGAG AGATC GATGA -3’ (antisense).

cDNA PKCe sequencing

The coding PKCe sequence (ENST00000306156; cDNA 11->397) was amplified from the cDNA as described above and the amplicons underwent direct sequencing analysis performed using the BigDye Terminator Cycle sequencing kit V 3.1 (Applied Biosystems) on a 3130Xl Genetic Analyzer (Applied Biosystems), following the manufacturer’s directions. Electropherograms were analysed using the SeqScape Software (Applied Biosystems) and sequences were blasted using the CLUSTAL W algorithm (www.ebi.ac.uk/clustalw).

Quantitative analysis for PKCe gene expression by Real-Time PCR

Equal quantities of RNA for each sample were retro-transcribed with Malone Murine Leukemia Virus Reverse Transcriptase (Promega, Madison WI, USA) according to manufacturer’s instructions. Two µl of 1:1 cDNA dilution were used to perform real-time PCR with GoTaq® qPCR master mix (Promega) and 200 nM of each primer in Applied Biosystems StepOne real-time machine (Applied Biosystems, Carlsbad, CA). Each reaction was performed in triplicate and mean Ct values were considered for quantitation. Relative gene expression was analysed using comparative Ct experiment software subset following manufacturer’s instructions.

The sequences of primers used for PCR were: CD41: 5’-GGAAT GTGCA GGGCT TTGAG-3’ (sense) and 5’-GGCTG TTCTT GCTCC GTATC-3’ (antisense); CD45: 5’-GGAAG TGCTG CAATG TGTCA TT-3’ (sense) and 5’-CTTGA CATGC ATACT ATTAT CTGAT GTCA-3’ (antisense); PKCe: 5’-CACCA TCCAG TTTGA GGAGC-3’ (sense) and 5’-CGACC CTGAG AGATC GATGA -3’ (antisense).

Table 1. Demographic characteristics and cardiovascular risk factors.

| Cardiovascular risk factors | MI N = 24 (%) | sCAD N = 24 (%) | HD N = 24 (%) |
|-----------------------------|--------------|-----------------|--------------|
| Mean age                    | 68.38        | 67.66           | 67.38        |
| Median (50 percentile)      | 68.0         | 68.5            | 69.5         |
| Gender                      |              |                 |              |
| -Male                       | 18 (75.00)   | 16 (66.67)      | 16 (66.67)   |
| -Female                     | 6 (25.00)    | 8 (33.33)       | 8 (33.33)    |
| Family history of cardiovascular disease |            |                 |              |
| -Yes                        | 7 (29.17)    | 11 (45.84)      | 4 (16.67)    |
| -No                         | 17 (70.83)   | 13 (54.16)      | 20 (83.33)   |
| Diabetes                    |              |                 |              |
| -Yes                        | 3 (12.50)    | 5 (20.83)       | 1 (4.16)     |
| -No                         | 21 (87.50)   | 19 (79.17)      | 23 (95.84)   |
| Smoking                     |              |                 |              |
| -Yes                        | 20 (83.33)   | 15 (62.50)      | 11 (45.83)   |
| -No                         | 4 (16.66)    | 9 (37.50)       | 13 (54.17)   |
| Hypertension                |              |                 |              |
| -Yes                        | 13 (54.16)   | 19 (79.17)      | 14 (58.33)   |
| -No                         | 11 (45.83)   | 5 (20.83)       | 10 (41.66)   |
| Body mass index             |              |                 |              |
| -normal                     | 9 (37.50)    | 10 (41.66)      | 10 (41.66)   |
| -pre-obese                  | 14 (58.33)   | 12 (50.00)      | 12(50.00)    |
| -obese                      | 1 (4.16)     | 2 (8.33)        | 2 (8.33)     |
| Hypercholesterolemia        |              |                 |              |
| -Yes                        | 8 (33.33)    | 17 (70.84)      | 5 (20.83)    |
| -No                         | 16 (66.66)   | 7 (29.16)       | 19 (79.16)   |

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Protein extraction and western blot

Total proteins were extracted from 1 ml (450×10^3/µl) of purified platelets. Briefly, 1 ml of isolated platelets from each sample was collected and centrifuged at 1700 rpm for 10 min. The pellets were then suspended in a cell lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na3VO4; 1 mM NaF) supplemented with fresh protease inhibitors and protein concentration was determined using BCA™ protein assay kit (Pierce, Rockford, IL). Fifty µg of proteins from each sample were then migrated in 5% SDS-acrylamide gels and blotted onto nitrocellulose filters.
Blotted filters were blocked and incubated with specific primary antibodies diluted as described in manufacturers’ protocols. Specifically, rabbit polyclonal anti-PKCε (Upstate, Lake Placid, NY) and mAb anti-β-actin (Sigma, Saint Luis, MO) were diluted 1:5000. Filters were washed and further incubated for 1.5 hours at room temperature with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase-conjugated anti-mouse IgG (Pierce) in the primary antibody working solution at RT. Specific reactions were revealed with the ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce).

PKCε protein expression in platelets by flow cytometry

Aliquots of PRP samples were stained with: i) anti-CD41a monoclonal antibody (mAb), as a marker of platelet population; ii) Thiazole Orange (TO), to identify reticulated platelets; iii) rabbit anti-PKCε monoclonal antibody, to test the presence of PKCε protein; and analyzed by flow cytometry. Briefly, 200 μl of PRP were washed and treated with IntraPrepTM Permeabilization Reagent (Immunotech, Marseille, France), following manufacturer’s protocol. One-hundred μl of Reagent 1 (fixation reagent) were added to the samples. After 15 min of incubation at room temperature, samples were washed and incubated with 100 μl of Reagent 2 (permeabilization reagent), 10 μl CD41a-Cy5 (Becton Dickinson, San Diego, CA) and 1 μl of affinity purified rabbit anti-PKCε Ab (Novus Biologicals, Littelton, CO); negative control was incubated with 100 μl of Reagent 2, 10 μl isotype-matched IgG-Cy5 (Immunotech) and 1 μl of an isotype-matched Ab (rabbit IgG, Sigma) for 45 min at room temperature in the dark. After a washing step, 1 μl of affinity purified goat anti-rabbit-PE Ab (Beckman Coulter) was added and the samples were incubated for 45 min at room temperature in the dark. Finally, the samples were washed and a solution 10 ng/ml of TO (Sigma) was added, in the presence or absence of 10 mg/ml RNAsi (negative control), and incubated for 30 min at room temperature in the dark and analysed by Flow Cytometry. Working dilutions of all reagents were previously determined with serial dilution tests. All samples were analysed on an FC500 flow cytometer (Beckman Coulter).

Platelet transfection with PKCε protein

Human recombinant PKCε (rPKCε; GenWay Biotech, San Diego, CA) was transfected into purified platelets using Proteojuice protein transfection reagent (Novagen, Podenzano, Italy), according to manufacturer’s protocols. For each transfection, 1 ml of PRP was centrifuged at 1800 rpm for 7 min, the supernatant was removed and pellets were washed with serum-free medium. Subsequently, samples were centrifuged at 1800 rpm for 7 min and medium was completely removed. The transfection mixture was prepared as follows: 25 μl of serum-free medium were added to 1 μg of PKCε protein (or nothing, as negative control) and 1.25 μl of ProteoJuice protein transfection reagent. After 20 min of incubation at room temperature, 225 μl of serum-free medium were added to the transfection mixture. Finally, the platelet samples were incubated with the mix at 37°C, 5% CO2 for 3.5 hours. After transfection, the samples were washed twice with serum-free medium to remove excess protein.

In some experiments, transfected and control platelets were re-added to platelet-deprived whole blood and aliquots were then used for platelet activation analysis by flow cytometry, as described above.

Platelet adhesion analysis in shear rate system

Isolated PRP was treated for rPKCε transfection, as described above. The procedures to prepare a washed erythrocyte suspension have been described previously in detail [39]. PRP containing 2–8×10^8 loaded platelets/ml was mixed with washed erythrocytes, to obtain a suspension with hematocrit of 42–45%, and apyrase (grade III; 142 ATPase U/mg of protein; Sigma) was added at the final concentration of 5 ATPase U/ml. The mixture was centrifuged at 1000 g for 15 min, the supernatant was discarded and the cell pellet was suspended in plasma. Suspensions of acid-insoluble fibrillar type I collagen from bovine achille tendon (Sigma) in 0.5M acetic acid (pH 2.8) were prepared as previously described [40] and two hundred microliters was used to coat glass coverslips for 60 minutes at 22–25°C in a humidified box. After coating, coverslips were washed with PBS and kept in a moist environment until assembled in a modified Hele-Shaw flow chamber [41]. The flow chamber was positioned on the stage of an inverted microscope equipped with epifluorescent illumination (Diaphot-TMD; Nikon Insech, Shinagawa-ku, Japan), an intensified CCD videocamera (C-2400-07, Hamamatsu Photonics, Shizhuoka, Japan), and appropriate filters. The total area of an optical field corresponded to approximately 0.007 mm^2. Blood cells were aspirated through the chamber with a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate calculated to obtain the desired wall shear rate at the inlet. Platelet adhesion was measured using blood containing 10 μM neparicin to render platelets fluorescent.

Three negative controls were run for each sample: i) untreated platelets; ii) platelets treated with a PKCε traslocation inhibitor peptide (Merck KgaA, Darmstadt, Germany) added to the transfection mixture (1 μg of inhibitor peptide) with or, iii) without rPKCε. Experiments were recorded in real time on videotape at the rate of 25 frames/s, which resulted in a time resolution of 0.04 s. Selected video sequences were also digitized in real time using a Matrox-Digisuite board (Matrox Graphics Inc., Dorval, Quebec, Canada).

Single frame images were captured from videotapes after an initial blood perfusion for 3 minutes on the different substrates. A threshold was applied to distinguish platelets from the background and the area occupied by all platelets in an image was measured using MicroImage (image-processing software; Tesi-Imaging srl, Venice, Italy).

Statistical analysis

The variables were compared between the three groups of patients using One Way Anova and Bonferroni t-test for multiple comparisons. Where indicated the variables were compared using Mann-Whitney test. T-test for independent or correlated samples was used to compare some indicated data. All the results are expressed as means plus or minus SD. Chi-square analysis of contingency tables were used for PKCε mRNA expression analysis of frequency comparison in the three groups. All the statistical tests were performed at the 0.05 p-value.

Results

MI patients carry hyper-responsive platelets

It is well known that patients with acute coronary syndromes carry hyper-responsive platelets [42,43] and show systemic platelet activation [43–47]. We therefore first studied the activation state of platelets from a randomly selected sub-group of our patients and their sensitivity to agonistic stimuli. In agreement with the current literature, we found that platelets from MI patients express significantly higher surface levels of p-selectin than those from sCAD patients and healthy donors (Fig. 1A). Moreover, in MI patients CD62p expression was induced at significantly higher levels by sub-optimal doses of ADP (Fig. 1B).
Platelets from MI patients contain mRNA for PKCe and express PKCe.

Since PKCe is finely modulated during megakaryopoiesis [34] and is known to increase the response of mouse platelets to GPVI-mediated activation [24], we reasoned that it could be a good candidate to account for the hyper-responsiveness of platelets from MI patients. Therefore, we extracted total mRNA from highly purified platelets and studied the expression of mRNA for PKCe by RT-PCR. The absence of nucleated cells contamination in the isolated platelet population was assessed by RT-PCR for CD45 expression. Moreover, given the dimensions (190 Kb) of the first intronic sequence of the PKCe gene and the positioning of the primers we use for PKCe RT-PCR, we could reasonably exclude the presence of PKCe pre-mRNA in platelets. In agreement with previous data [24], expression of PKCe mRNA was found at low frequency both in platelets from normal subjects and sCAD patients. On the contrary, platelets from the majority of MI patients (21 out of 24; 87.5%) had a clear-cut expression of PKCe mRNA, as confirmed also by qPCR (Fig. 2D). Retroscribed cDNA from platelet PKCe mRNA (MI patients) was bi-directionally sequenced in the region encompassing the exons 1 and 2 (ENST00000306156; nucleotides from 11 to 307). Analysis of the sequences and subsequent BLAST analysis revealed a truly concordance of cDNA with PKCe mRNA (encompassing the first intronic sequence of the PKCe gene and the positioning of the primers we use for PKCe RT-PCR, we could reasonably exclude the presence of PKCe pre-mRNA in platelets). In agreement with previous data [24], expression of PKCe mRNA was found at low frequency both in platelets from normal subjects and sCAD patients. On the contrary, platelets from the majority of MI patients (21 out of 24; 87.5%) had a clear-cut expression of PKCe mRNA, as confirmed also by qPCR (Fig. 2D).

Figure 1. Platelet activation in MI patients. Panel A: Left. Flow cytometric analysis of platelet CD62p surface expression in patients with MI, sCAD and healthy donors. Cells were stained with specific mAb anti P-selectin, as described in Materials and Methods. Ten patients were analyzed for each group. Right, Quantification of CD62p expression on the surface of platelets from MI, sCAD and HD. Absolute numbers of surface antigens expressed/cell (MESF). (Anova and Bonferroni t-test; * P<0.05 MI vs HD; no significant differences were found between stableCAD and HD). Panel B: Platelet surface expression of CD62p in MI ( ● ), sCAD ( ▲ ) and HD ( ○ ) patients in the presence of increasing doses of ADP. Left panel shows the percentage of positive cells. Right panel shows the absolute numbers of surface antigens expressed/cell (MESF). Data from 7 patients of each group are expressed as means ± S.D. (Anova and Bonferroni t-test; * P<0.05 MI vs HD; no significant differences were found between stable CAD and HD).

To test if this “ectopic” expression of PKCe in the platelets of MI patients could be attributed to the immature platelet fraction, we analysed by flow cytometry the population of reticulated platelets from MI patients and HD. As reported in Figure 3C, the percentage of platelets from MI patients expressing PKCe protein was significantly increased both in mature (TO-EPS+) and immature (reticulated) (TO+EPS+) platelet fractions, as compared to HD. More specifically, the ratio between PKCe positive platelet subsets from MI vs HD subjects was 2.9±0.73 in mature platelets (TO-EPS+) and 2.17±1.44 in immature platelets (TO+EPS+) (p = 0.48, ns).

Platelets from MI patients during follow up become negative for PKCe mRNA

To test whether the expression of PKCe in MI was transient or stable, 11 randomly selected MI patients positive for platelet PKCe mRNA expression were re-called between day 15 and day 30 from the acute episode and the platelets were tested again for PKCe expression. All platelet samples were found negative for PKCe mRNA expression as soon as 15 days from the acute MI episode (not shown).

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PKCe-expressing platelets are hyper-responsive

To study the correlation between PKCe expression and platelet activation, we compared the p-selectin (CD62p) cell surface expression in PKCe positive vs PKCe negative platelets in all the analysed groups (Fig. 4A). The general trend is a significantly higher p-selectin expression in PKCe positive cells. Subsequently, we tested the platelet reactivity to the stimulation with sub-optimal doses of ADP. As expected, both PKCe negative and positive platelets were activated, as compared to controls (resting), but the
positive cells were significantly more reactive than the negative (Fig. 4B).

PKCε-overexpressing platelets are hyper-responsive and show enhanced adhesion to collagen.

To study the functional role of PKCε in platelets, we subsequently decided to force its expression in platelets from normal healthy donors, in vitro mimicking the in vivo situation. rhPKCε protein was therefore successfully transfected in healthy donor platelets (thus originally negative for PKCε expression) (Fig. 5A). Subsequently, PKCε⁺ platelets were functionally assayed for ADP-induced activation and shear stress adhesion, using mock-transfected normal platelets as negative controls.
Figure 3. Platelets from MI patients express PKC\(\varepsilon\) protein. Panel A: A representative Western blot assay for the detection of total PKC\(\varepsilon\) protein expression in platelets from PKC\(\varepsilon\) mRNA-positive MI, sCAD and HD. \(\beta\)-Actin was assayed for protein loading. Panel B: Densitometric analysis of PKC\(\varepsilon\) protein expression normalized against \(\beta\)-actin in PKC\(\varepsilon\) mRNA-positive MI, sCAD and HD platelets. Data are expressed as means \pm S.D. (Anova and Bonferroni t-test; * P<0.05 MI vs HD; \# P<0.05 MI vs sCAD; no significant differences were found between sCAD and HD). Panel C: PKC\(\varepsilon\) protein expression in mature and immature platelets from healthy donors (HD) and MI patients. Cells were simultaneously labelled with Thiazole Orange (TO) - to visualize the immature platelet fraction (IPF) - and anti-PKC\(\varepsilon\) mAb, and analyzed by flow cytometry. Three populations were identified within the CD41+ cell subset: PKC\(\varepsilon\) negative reticulated platelets (TO–EPS+); PKC\(\varepsilon\) positive reticulated platelets (TO–EPS–); PKC\(\varepsilon\) positive mature platelets (TO–EPS+). (Data from 3 patients/group, expressed as means \pm S.D. t-Test ** P<0.001; * P<0.05). doi:10.1371/journal.pone.0046409.g003

Figure 4. PKC\(\varepsilon\) protein expression in platelets correlates with their activation levels. Panel A: Flow cytometric analysis of platelet CD62p surface expression in patients with MI, sCAD, healthy donor and in all the sample (ALL), on the basis of PKC\(\varepsilon\) expression. Cells were stained with specific mAb anti P-selectin (CD62p). Seven patients were analyzed for each group (MI: 2 PKC\(\varepsilon\) negative and 5 PKC\(\varepsilon\) positive samples; sCAD: 4 PKC\(\varepsilon\) negative and 3 PKC\(\varepsilon\) positive samples; HD: 4 PKC\(\varepsilon\) negative and 3 PKC\(\varepsilon\) positive samples; ALL: 4 PKC\(\varepsilon\) negative and 3 PKC\(\varepsilon\) positive samples). Data is expressed as mean \pm S.D (Anova and Bonferroni t-test). Panel B: Flow cytometric analysis of CD62p surface expression in PKC\(\varepsilon\) negative and positive platelets. Cells were treated with ADP and compared with untreated platelets (resting). Ten patients were analyzed for each group. Data is expressed as mean \pm S.D (Anova and Bonferroni t-test). doi:10.1371/journal.pone.0046409.g004
First, PKCε normal platelets and control platelets were treated with minimal doses of ADP (0.3 μM) and analyzed for CD62p surface expression by flow cytometry. There were no significant differences in the surface expression of CD62p between PKCε+ normal platelets and control platelets in the absence of ADP stimulation. On the contrary, PKCε+ platelets treated with 0.3 μM ADP express significantly higher levels of CD62p than control platelets (Fig. 5B).

Second, we studied the adhesion to collagen-coated surfaces of PKCε-overexpressing normal platelets under controlled physiological low (600 sec⁻¹) or high (1500 sec⁻¹) shear rates. Adhesion to collagen at 1500 sec⁻¹ was significantly increased in normal platelets over-expressing PKCε, particularly after 3 minutes testing (Fig. 6). Although hampered by a high variability, adhesion at low shear rate also showed a trend to increase. Adhesion of PKCε-transfected platelets treated with the PKCε inhibitor was similar to that of untreated platelets, demonstrating the specificity of the observed enhancement.

**Discussion**

Platelets play a central role in the genesis and propagation of atherothrombosis and are therefore the target of several therapies for the prevention of thrombosis, particularly in the coronary artery district, where thrombi are the main responsible for myocardial infarction. Platelets are produced by megakaryocytes as anucleated cells that however retain the protein synthesis machinery, some small pre-mRNAs and selective mRNAs that can therefore be efficiently translated during the platelet life, that lasts around 10 days.

In general, PKC is established as an important regulator of several platelet functions. Of the many known PKC isoforms, not all are physiologically expressed in mature platelets, with some important differences between human and mouse platelets. It is now possible however to associate specific platelet functions to specific PKC isoforms. For instance, Konopatskaya et al. [48] recently demonstrated, using a genetic approach, the key role played by PKCα in α-granules release and thrombus formation, with no role in platelet adhesion to collagen. On the contrary, absence of PKCδ and PKCζ enhances the activation response to GPVI agonists and adhesion to collagen [46]. Moreover, the absence of PKCβ and θ abrogate outside-in signaling via αIIbβ3 integrin [11,23]. Indeed, several receptors involved in platelet activation signal via PKC family members, like – for instance – thrombin or the collagen receptor GPVI. As a consequence, pharmacological inhibitors of PKC inhibit platelet aggregation.

In our hands, the majority of HD did not express PKCε in their platelets. This is in agreement with our previous data [34] describing the down-modulation of PKCε expression in in vivo human megakaryocytopenia from day 5 onward of TPO-driven MK differentiation of CD34 precursors. It is interesting however that mouse platelets – on the contrary – express PKCε at high levels. From the studies in mice we learn that PKCζ plays a relevant role in the activatory signaling cascade emanating from the GPVI receptor. In fact, Pears et al. [24] elegantly demon-
Figure 6. PKCε enhances platelet adhesion to fibrillar type I collagen under flow conditions. rhPKCε-transfected and control platelets were reconstituted in whole blood, previously deprived from PRP, and tested for their adhesion capacity under flow. Mepacrine-loaded platelets (5–7 x 10^11/ml) and washed erythrocytes (hematocrit 42–45%) suspended in plasma, were perfused for 3 minutes over immobilized fibrillar type I collagen. Surface coverage was measured on an area of 0.07 mm^2 after 3 minutes of perfusion at 600 s⁻¹ or 1500 s⁻¹ and is shown as mean ± 95% confidence intervals of at least 3 separate experiments. Results are shown relative to the values observed in untreated blood cell suspensions (control) (Anova and Bonferroni t-test; * p < 0.01). Representative single-frame images of each surface are also shown. doi:10.1371/journal.pone.0046409.g006

...strated a marked reduction in the onset of aggregation and level of ATP secretion in response to collagen in the PKCε null mouse in the absence of collagen receptors alterations. In particular, the same Authors identify a role for PKCε in the GPVI signaling pathway, mediated by a reduction of FcγRII-chain phosphorylation.

The development of a platelet thrombus in coronary arteries is usually a critical, final phase in atherothrombosis, leading to MI. At the high shear rates typical of small arteries, the initial tethering of platelets at the sites of vascular injury or plaque rupture is mediated by GPIb/V/IX and collagen-immobilized VWF. This interaction – however – has a high off rate, and is not sufficient for stable adhesion and platelet activation, that are the key events for the formation of the thrombus. The shift to stable adhesion requires the subsequent interaction between the platelet and the extracellular matrix proteins, notably between GPVI and collagen [49–51]. GPVI is a signal-transducing, non-integrin collagen receptor that mediates platelet activation, secretion of pro-coagulative factors and surface phosphatidylserine expression that promotes thrombin formation. Interestingly, Bigalke et al. recently demonstrated that platelet surface GPVI expression is already elevated hours before the onset of MI [50]. Genome wide association studies analyzed the GPVI gene in detail, finding large differences in haplotypes which might account for the substantial inter-individual variation in the platelet response to collagen (or to Collagen Related Protein, CRP). Subsequent proteomic studies performed in high, mid and low responders showed something like 1,000 proteins, clustered in patterns, which included signaling as well as trafficking and transmembrane proteins [52]. We show that human platelet PKCε is selectively de novo expressed in MI, but not in sCAD patients, during the acute event while its expression returns negative after 15 days of follow-up. Functionally, we demonstrate that PKCε-transfected normal human platelets enhance their adhesion properties to collagen-coated surfaces under physiologically high shear forces. MI patients express PKCε mRNA at significantly higher frequency than HD and sCAD. Considering the dimensions of the first intronic sequence of the PKCε gene, that would virtually preclude the persistence of a potential PKCε pre-mRNA in the platelet, our findings suggest that platelet generations produced before the acute event of MI might retain PKCε-mRNA that is not down-regulated during terminal MK differentiation. An alternative explanation would be an anticipated release of platelets, before physiological PKCε down-modulation. This possibility is however unlikely, as PKCε down-modulation takes place around day 6 of in vitro MK differentiation, that would be too early. Besides, the analysis conducted on the reticulated platelets of some MI patients of our cohort did not show any difference in terms of of PKCε expression as compared to mature platelets, excluding the possibility that the appearance of PKCε positive platelets in MI patients could be selectively ascribed to newly formed platelets.

As a novel PKC isoform, PKCε would then boost platelet activation responding to DAG generation downstream the immunoreceptor GPIV-FcRγ complex, that signals via cytoplasmic PLCγ, or any other activating surface receptor that signals via phosphoinositides breakdown. To this respect, one could speculate that an ectopic expression of PKCε accelerates platelet activation and perhaps the inside-out signaling that is essential to form thrombi. From a different perspective, the ectopic expression of PKCε in platelets could be used as a marker of probability to anticipate the acute event in patients at risk.

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Author Contributions

Conceived and designed the experiments: GG PM MV. Performed the experiments: CC MM DG MRC LDM NM MG. Analyzed the data: GG PM MV CC DG. Contributed reagents/materials/analysis tools: MM DG MRC LDM NM MG FN DL. Wrote the paper: GG MV. Selected the patients and organized the clinical work: DA PAM. Treated the patients: DL FN.
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