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Journal of Cellular Biochemistry 111:889–898 (2010)

Journal of Cellular Biochemistry

Effects of Platelets on the Protein Expression in Aortic Segments: A Proteomic Approach

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ABSTRACT

It is well known the effects of the vascular wall on platelet activity but little is known about the effects of platelets on the proteins expression in the vascular wall. We analyzed whether platelets may modify the protein expression in the vascular wall. We used an in vitro model coincubating human platelet rich plasma (PRP) with control and 10 ng/ml tumor necrosis factor-α (TNF-α)-preincubated bovine aortic segments. 2DE, mass spectrometry and Western blot analysis were used to determine changes in the expression of proteins associated with the cytoskeleton and energetic metabolism in the aortic segments. In control healthy vascular wall, only the cytoskeleton-related proteins expression was modified by PRP. However, when PRP was coincubated with TNF-α pre-stimulated aortic segments lesser number of cytoskeleton-related proteins were modified. With respect to energetic metabolism, in control segments, PRP failed to modify any of the analyzed energetic-related proteins. However, in TNF-α-preincubated segments the presence of PRP upexpressed glyceraldehyde-3-phosphate dehydrogenase. Moreover, by western blot experiments it was observed that in TNF-α-preincubated segments the expression of fructose 1,6-bisphosphate aldolase was downregulated by platelets. However, no differences were found in the expression of triosephosphate isomerase and ATP synthase α-chain. In addition, the activity of fructose 1,6-bisphosphate aldolase and piruvate content was significantly reduced without modification on triosephosphate isomerase activity. In conclusion, the crosstalk between platelets and vascular wall is bidirectional and platelets regulated in the vascular wall the expression of proteins associated with the cytoskeleton and energetic metabolism, particularly in the healthy vascular wall. J. Cell. Biochem. 111: 889–898, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: VASCULAR DISEASE; THROMBOSIS; PLATELETS AND PROTEOMICS

Not long ago, the concept of platelets in atherosclerosis was that of a cell belonging to the coagulation cascade and participating in the final step of atherosclerosis, the rupture of the plaque followed by thrombotic narrowing or occlusion of a vessel [Theilmeier et al., 2002; Jorgensen, 2005]. However, new insights into the pathogenesis of atherosclerosis and atherothrombosis have been changing this dogma.

It has been extensively demonstrated that platelet activity is modulated by factors expressed and released from the vascular wall [Bombeli et al., 1998; Massberg et al., 1998]. Indeed, healthy vascular wall represents a non-adhesive and non-thrombotic surface that inhibits platelet activity [Jaffe, 1987; Cines et al., 1998], while, under inflammatory conditions, as it occur in arterial ischemic events, the antithrombotic properties of the vascular wall shift towards a prothrombotic state favoring platelet activation [Hawiger, 1987; Catella-Lawson, 2001].

Although much is known about the effects of the vascular wall on platelets [Catella-Lawson, 2001], studies examining the effect of platelets on the expression of proteins in the vascular wall are sparse. Platelets are source of several mediators including growth

There are no conflicts of interest in relation with the present study.

Grant sponsor: Fondo de Investigaciones de la Seguridad Social (Redes temáticas de Cooperación Red Heracles); Grant number: RD06/0009/010; Grant sponsor: Fondo de Investigaciones de la Seguridad Social; Grant number: FIS04/1205.

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Received 20 October 2009; Accepted 8 July 2010 • DOI 10.1002/jcb.22777 • © 2010 Wiley-Liss, Inc.

Published online 21 July 2010 in Wiley Oline Library (wileyonlinelibrary.com).

factors and other substances that may influence the expression of proteins in the vascular wall [Massberg et al., 2003]. In this regard, in vitro studies have suggested that platelets may induce inflammation on culture endothelial cells [Gawaz et al., 1998; Catella-Lawson, 2001]. In vivo studies have also demonstrated that, under vascular inflammatory conditions, platelets may modulate the expression of inflammatory-associated proteins, such as the inducible nitric oxide synthase [González-Fernández et al., 1998].

It is well known that disturbances in vascular function contribute to the development of the cardiovascular diseases. In this regard, further to the control of inflammation, there is a large body of evidence suggesting that the maintenance of normal vascular function is particularly dependent of energy metabolism [Paul, 1989]. Both contractile reactivity and vasorelaxation have been reported to be specifically dependent of glucose metabolism [Zhang and Paul, 1996]. Interestedly, during the process of energy production in aerobic physiological conditions vascular cells produce reactive oxygen species, which means to count with a potent antioxidant system. The cytoskeleton and contractile system are also another critical components of the vascular wall and they directly contribute to modulate arterial haemodynamics and to maintain vascular integrity [Herman et al., 1987]. Vascular structure, including the vascular cytoskeleton, seem to be regulated by the arterial mechanical environment [Zhao et al., 1995]. However, other factors and neighboring vascular cells, such as platelets, may be also involved in such regulation. Therefore, the crosstalk between the cells contained in the vascular wall and platelets could be more complex than that we initially may believe and the relationship between platelets and the vascular wall seem to deserve new investigations.

Until now, it has been difficult to monitor changes in the expression of several proteins at the same time in a single sample. A technology termed as proteomics, which it is based in the use of two dimensional electrophoresis (2-DE) and mass spectrometry analysis has emerged providing us a useful methodology to quantify and identify changes in the expression of multiple proteins and proteins isotypes in a single sample. The aim of the present study was then to analyze, using proteomics, whether platelets may modified the expression of proteins in the vascular wall particularly those associated with vascular cytoskeleton and energetic metabolism. For this purpose, we used an in vitro model coincubating platelet rich plasma with isolated healthy aortic segments and with aortic segments submitted to a pre-inflammatory situation prior to be coincubated with the platelets.

MATERIALS AND METHODS

ISOLATION OF HUMAN PLATELET-RICH PLASMA (PRP) AND COINCUBATION WITH BOVINE AORTIC SEGMENTS

Human platelet-rich plasma (PRP) was obtained from 35 ± 8 years old healthy volunteers who were not taken aspirin or any other anti-platelet of anti-inflammatory drugs from at least 15 days before blood extraction. As previously described, PRP was prepared from whole blood obtained in 10% (v/v) acid-citrate-dextrose and centrifuged at 800*g* for 15 min at 20°C. PRP was collected and the platelet number was counted by a coulter counter. When it was necessary, platelet poor plasma was used to dilute PRP.

Bovine thoracic aortic segments were used for the coincubation experiments. The thoracic aortic segments were carefully isolated to preserve the endothelium, cut into portions (~1 cm each) and then they were preincubated in RPMI medium containing 1% fetal calf serum, 5 mmol/L glutamine, 2×10^{-5} U/L penicillin, and 2×10^{-5} μg/L streptomycin during 1 h. Bovine aortic segments were then washed twice and coincubated with PRP. In same experiment before coincubation with PRP, bovine aortic segments were stimulated with tumor necrosis factor-α (TNF-α) 10 ng/ml for 6 h [Zamorano-León et al., 2006]. After TNF-α incubation, the aortic segments were twice washed and then coincubated with PRP, therefore, only the aortic segments were preincubated with TNF-α.

Two different platelet concentrations (10^5 and 10^7 platelets/well) were used in the coincubation experiments. The final platelet concentration was obtained by diluting PRP with platelet poor plasma (PPP). In this regard, 10^5 platelets were obtained from diluting 10^7 platelets with PPP. PPP was obtained by centrifugation of PRP at 2,500 rpm for 10 min. The aortic segments used as control were incubated with the same amount of PPP than the final volume of PRP + PPP added in the aortic segments incubated with 10^5 platelets/well.

The coincubation system was prepared by placing sterile Transwell-COL (Corning incorporated, COSTAR 3491, NY) inserts of 24 mm diameter and 0.4 μm pore size, containing the platelets, into wells containing the bovine aortic segments (Corning incorporated, COSTAR 3491). The pore size used in the coincubating experiments prevents the migration of platelets into the lower chamber that contained the bovine aortic segments, since platelets have an average diameter of 1–3 μm [Bain, 2002].

In the coincubation system, the medium was shared by both platelets and the aortic segments and it does possible the further processing of the isolated vascular segments alone [Molero et al., 2003]. After 1 h of coincubation, the transwell inserts containing the platelets were removed and the isolated aortic segments were incubated at 37°C for further 23 h and then recovered and frozen quickly at −80°C for the proteomic analysis. The fact that PRP was only coincubated during 1 h with the isolated aortic segments was based in previous observations that times longer than 1 h favored that platelets were spontaneously activated [López-Farré et al., 1990]. All the experiments were performed in RPMI medium containing 1% fetal calf serum, 5 mmol/L glutamine 2×10^{-5} U/L penicillin and 2×10^{-5} g/L streptomycin.

TWO-DIMENSIONAL ELECTROPHORESIS

Bovine aortic segments were homogenized with an Ultra-Turrax T8 IKA-Werke homogenized in a buffer containing 8 mol/L urea, 2% CHAPS w/v, 40 mmol/L dithiothreitol, 0.2% Bio-LyteTH ampholyte (Bio-Rad Laboratories) and 0.01% w/v bromophenol blue. The homogenate tissues were centrifuged at 10,000*g* for 10 min and the supernatant stored at −80°C until further analysis. Protein concentration was estimated by bicinchoninic acid reagent (Pierce), using bovine serum albumin as standard. Two hundred fifty micrograms of total protein was loaded on immobilized gradient IPG strips (pH 3–10) and isoelectric focusing was performed using a

Protean IEF cell system (Bio-Rad Laboratories) as reported [Molero et al., 2005]. In brief, the gels were actively rehydrated at 50 V for 60 h, then rapid and linear voltage ramping steps, limited by a maximum current of 50 μA per gel, were applied [Mateos-Cáceres et al., 2004]. In the second dimension, the proteins from the strips were resolved on 10% SDS–PAGE gels using a Protean II XL system (Bio-Rad Laboratories). Afterwards, the gels were fixed and silver stained. The silver staining was developed as described using a Silver Stain Plus Kit (Bio-Rad Laboratories).

IMAGE ACQUISITION AND ANALYSIS

The stained gels were scanned in a UMAX POWERLOOK III Scanner operated by the software Magic Scan V 4.5. Intensity calibration was carried out using an intensity stepwedge before gel image capture. Image analysis was carried out using Quantity One 4.2.3 (Bio-Rad Laboratories). Image spots were initially detected, matched, and then manually edited. Each spot intensity volume was processed by background subtraction.

MASS SPECTROMETRY

To identify the spots of interest, they were manually excised from the gels using biopsy punches and spots underwent mass spectrometry. Proteins selected for analysis were digested with trypsin as previously reported [Mateos-Cáceres et al., 2004]. After digestion, the peptides were extracted with 100 mmol/L ammonium bicarbonate and then, the extracts were lyophilized and resuspended in 0.1% TFA. The peptides were purified by use of a mmol/l–18 Zip tips (Millipore). For mass spectrometry (MS) analysis, 1 μl of purified extracts was mixed with a 1 μl of α-cyano-4-hydroxy-trans cinnamic matrix (Sigma) in 50% acetonitrile and 1 μl of this mixture was spotted on to a Maldi plate and allowed to air-dry at room temperature. MS analyses were performed in a 4700 Proteomic Analyzer (Applied Biosystems) and operated in a reflector positive mode. All mass spectra were calibrated using a standard peptide mixture (Applied Biosystems). The analysis in a MS mode produced peptide mass fingerprints, and some peptides observed were further analyzed in a MS/MS mode as reported previously [Mateos-Cáceres et al., 2004]. Peptides with a signal-to-noise greater than 20 were considered in the Mascot Database for protein identification. For protein identification, Mascot database 1.9 (http://www.matrixscience.com) was used as algorithm to match the peptides obtained by MS. The MS identifications were accepted based on a tripartite evaluation that takes into account significant molecular weight search (Mowse) scores, spectrum annotation and observed versus expected migration on the 2-DE gel.

WESTERN BLOT ANALYSIS

In the bovine aortic segments, the protein expression of triosephosphate isomerase, fructose 1, 6-bisphosphate aldolase and mitochondrial ATP synthase α-chain was analyzed by Western blot. In brief, the homogenized vascular segments were solubilized in Laemmli buffer containing 2-mercaptoethanol. The obtained proteins were separated on denaturing SDS–PAGE 15% (w/v) polyacrylamide gels. Equal amount of proteins (20 μg/lane), estimated by bicinchoninic acid reagent (Pierce), were loaded. To assess that the same amount of proteins were loaded into the gel, a parallel gel with identical samples was run and stained with Coomassie. Proteins were then blotted onto nitrocellulose (Immobilion-P; Millipore) and the blots were blocked overnight at 4°C with 5% (w/v) non fat dry milk in TBS-T mmol/L Tris/HCl (pH 5.2), 137 mmol/L NaCl and 0.1% Tween-20, as reported [Mateos-Cáceres et al., 2004]. The membranes were incubated with monoclonal antibodies against either triosephosphate isomerase (1:500; sc-30145, Santa Cruz Biotechnology), fructose 1,6-bisphosphate aldolase (1:500; Aldolase A (N-15): sc-12059, Santa Cruz Biotechnology) and mitochondrial ATP synthase α-chain (1:2000; ATP5A (15H4): sc-58613, Santa Cruz Biotechnology). Membranes were then incubated with HRP (horseradish peroxidase)-conjugated anti-(mouse, rabbit or goat IgG) antibodies at a dilution of 1:2,000. The proteins were detected by enhanced chemiluminescence (ECL®, Amersham Biosciences) and evaluated by densitometry (Quantity One; Bio-Rad Laboratories). Pre-stained protein markers (Sigma) were used for molecular mass determinations.

DETERMINATION OF THE FRUCTOSE 1,6-BISPHOSPHATE ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE ACTIVITIES

The activity of both fructose 1,6-bisphosphate aldolase and triosephosphate isomerase was determined in the vascular wall following the method described by Misset and Opperdoes [1984]. In brief, 240 μg of total protein from the vascular segments was incubated in 100 mmol/L Tris–HCl buffer pH 7.4 (T-1503, Sigma–Aldrich), 58 mmol/l fructose 1,6-diphosphate solution (F-0752, Sigma–Aldrich), 4.0 mmol/L β-nicotinamide adenine dinucleotide reduced form (β-NADH, N-8129, Sigma–Aldrich) and 50 U/ml of α-glycerophosphate dehydrogenase/triosephosphate isomerase (G6755, Sigma–Aldrich) for aldolase assay. The aldolase activity was assayed at 340 nm at 25°C using plastic cuvetts.

The same amount of total protein was used for the determination of triosephosphate isomerase activity. The vascular segments were incubated in 100 mmol/L Tris–HCl buffer pH 7.4 (T-1503, Sigma–Aldrich), 58 mmol/L glyceraldehyde-3-phosphate solution (G-5251, Sigma–Aldrich), 4.0 mmol/L β-nicotinamide adenine dinucleotide reduced form (β-NADH, N-8129, Sigma–Aldrich) and 50 U/ml of α-glycerophosphate dehydrogenase/aldolase (A-2714, Sigma–Aldrich). The triosephosphate isomerase activity was assayed at 340 nm at 25°C using plastic cuvetts.

DETERMINATION OF THE PYRUVATE CONTENT

The content of pyruvate present in the bovine aortic segments was quantified using a Pyruvate Assay Kit (K609-100, BioVision Research Products, USA). For this purpose, 80 μg of each homogenized platelet solution was used to assay pyruvate concentration using the colorimetric assay following the manufacturer's instructions.

STATISTICAL METHODS

Results are expressed as mean ± SEM. A Mann–Whitney test was performed to determinate the level of significance. A P value <0.05 was considered statistically significant.

RESULTS

In the bovine aortic proteomic map the spots were densitometrically analyzed and identified by comparison with those found in the human saphenous vein published by McGregor et al. [2001]. The spots in which statistical differences were observed were further identified by mass spectrometry (see Table I).

The present study was only focused in the analysis of proteins associated with two main biological functions: cytoskeleton and contractile system and energetic metabolism.

CHANGES BY PRP ON CYTOSKELETON AND CONTRACTILE SYSTEM-ASSOCIATED PROTEINS IN BOVINE AORTIC SEGMENTS

The following proteins associated with the vascular cytoskeleton and contractile system were identified: (a) microfibril-associated glycoprotein 4; (b) α-actinin associated LIM protein; (c) α-actin isotypes 1 and 2; (d) β-actin isotypes 1 and 2; (e) β-tubulin; (f) annexin A4; (g) annexin A2; (h) annexin A5 isotypes 1 and 2 (i) tropomyosin β-chain isotypes 1 and 2; and (j) SM22/transgelin (Fig. 1). In Table I is shown the experimental mass and isoelectric point (IP) of the proteins identified by mass spectrometry.

In the normal aortic segments, the presence of PRP significatively reduced the expression of α-actinin-associated LIM protein, α-actin isotype 2, β-tubulin, annexin A5 isotype 1, tropomyosin β chain isotype 1 and SM22/transgelin (Table II and Fig. 1). Most of the proteins were changed in their expression by both 10^5 and 10^7 platelets/well except for β-tubulin that it was only reduced by 10^7 platelets/well (Table II). In normal healthy vascular wall, 10^5 platelets/well and 10^7 platelets/well upexpressed β-actin isotype 1 and 10^7 platelets/well upexpressed microfibril-associated glycoprotein 4 (Table II and Fig. 1).

In the vascular wall, when platelets was coincubated with TNF-α pre-stimulated aortic segments the presence of PRP changed the expression of a lesser number of cytoskeleton and contractile-related proteins than in the normal healthy aortic segments (Table II). Indeed, in TNF-α-preincubated aortic segments the presence of PRP modified the expression of the following proteins: α-actin isotype 2, β actin isotype 1, Annexin A5 isotype 2, and tropomyosin β-chain isotype 1 (Table II). The protein expression of α-actin isotype 2 and β-actin isotype 1 was increased by the presence of PRP (Table II). However, the protein expression of annexin A5 isotype 2 and tropomyosin β-chain isotype 1 was reduced by PRP (Table II). The modification in the level of expression of both β-actin isotype 1 and annexin A5 isotype 2 in the TNF-α-incubated aortic segments was only observed with 10^7 platelets/well and did not with 10^5 platelets/well (Table II).

CHANGES IN THE EXPRESSION OF PROTEINS ASSOCIATED WITH ENERGETIC METABOLISM IN BOVINE AORTIC SEGMENTS COINCUBATED WITH PRP

In the vascular proteomic map the following proteins associated with the energetic metabolism were identified: (a) glyceraldehyde-3-phosphate dehydrogenase; (b) phosphoglycerate mutase; (c) two isotypes of mitochondrial aldehyde dehydrogenase; (d) ATP synthase β-chain; and (e) glutathione-S-transferase (Fig. 1). Table I shown the

TABLE I. Methods Followed to Identify Vascular Wall Proteins After 2-DE Electrophoresis

Protein	Database accession number	Theoretical mass (kDa/pI)	Peptides matched	Confirmation method	Protein score	Total ion score	Sequence coverage (%)
Annexin A2	P04272	38.74/6.9	QDIAFAYQR	MS/MS	–	14	2
α-Actin smooth muscle	P62736	42.38/5.23	AGFAGDDAPR/GYSFVTTAER/GYSFVTTAER/AVFPSIVGRPR/ AVFPSIVGRPR/QEYDEAGPSIVHR/SYELPDGQVITIGNER/ SYELPDGQVITIGNER	MS + MS/MS	111	70	15
α-Actin smooth muscle	P62736	42.38/5.23	GYSFVTTAER/GYSFVTTAER/AVFPSIVGRPR/AVFPSIVGRPR/ QEDEAGPSIVHR/IWHHSFYNELR/IWHHSFYNELR/SYELPDGQVITIGNER/ SYELPDGQVITIGNER/DLYANNVLSGGTTMYPGIADR	MS + MS/MS	49	13	21
β-Actin	P84336	42.05/5.29	AGFAGDDAPR/AVFPSIVGRPR/AVFPSIVGRPR/IWHHTFYNELR/ IWHHTFYNELR/SYELPDGQVITIGNER/SYELPDGQVITIGNER/ VAPEEHPVLLTEAPLNPK/VAPEEHPVLLTEAPLNPK/ DLYANTVLSGGTTMYPGIADR/DLYANTVLSGGTTMYPGIADR	MS + MS/MS	115	55	37
Tropomyosin β chain	P58776	32.93/4.6	IQLVEEELDR	MS/MS	–	34	3
Tropomyosin alpha chain	P58772	32.71/4.6	LVIIESDLER/IQLVEEELDR/KLVIIESDLER/RIQLVEEELDR	MS/MS	–	112	7
ATP synthase β-chain	Q9MEI6	81.18/9.76	KIYLPLLLPQQ	MS/MS	–	3	16
Mitochondrial aldehyde dehydrogenase	P12762	54.53/5.70	EDVDRAVK/YYAGWADK/TFPTVNPSTGEVICQVAAGDK/ KTFPTVNPSTGEVICQVAAGDK	MS	34	–	7

MS, mass spectrometry; MS/MS, tandem mass spectrometry. Database accession number represent the number assigned in the Mascot database 1.9 (http://www.matrixscience.com) used as algorithm to match the peptides obtained by mass spectrometry.

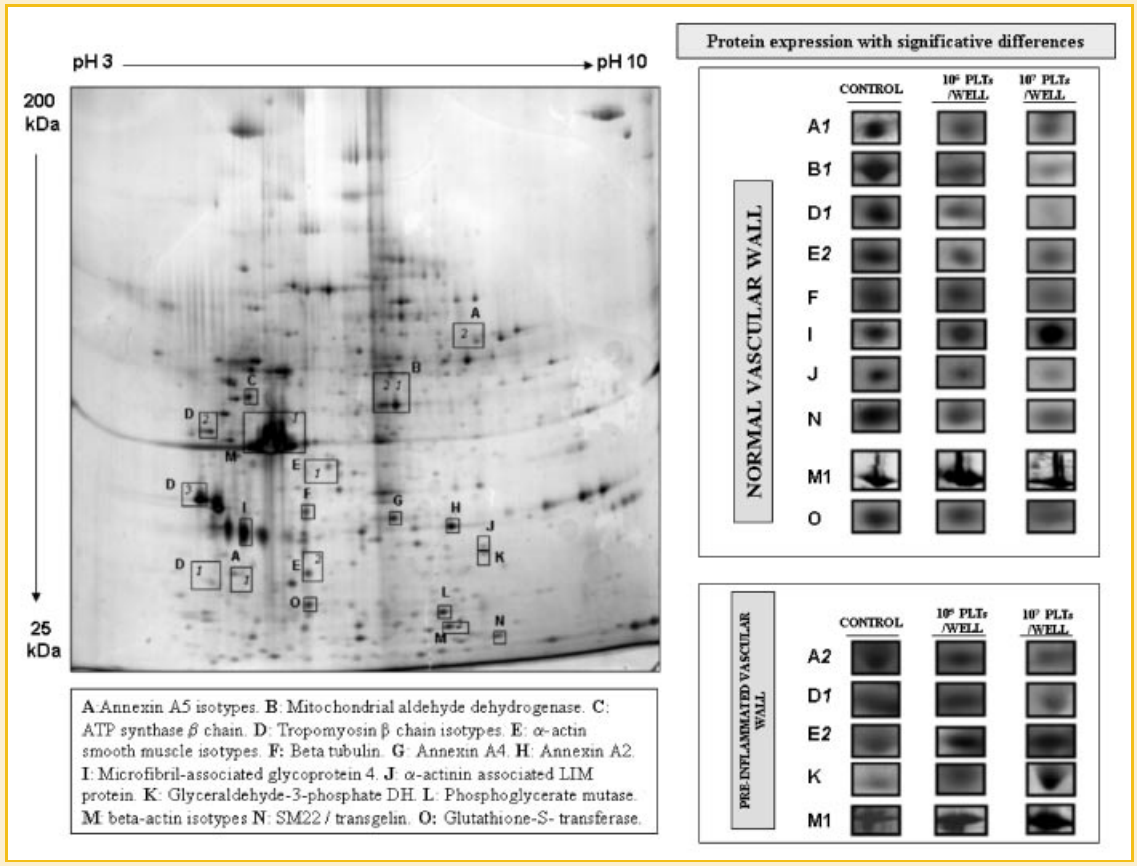

Fig. 1. Representative bidimensional gel electrophoresis (2-DE) of bovine aortic proteins in a pH range between 3 and 10. The bidimensional gel shows the different proteins analyzed in the proteomic study. Proteins were identified by comparison with the human saphenous vein published by McGregor et al. [2001].

experimental mass and IP of some of the above-mentioned proteins identified using mass spectrometry.

In normal healthy vascular wall, the presence of PRP reduced the protein expression of mitochondrial aldehyde dehydrogenase isotype 1 and glutathione-S-transferase (Table III and Fig. 1). The reduction of glutathione-S-transferase was only observed with the presence of 10^7 platelets/well (Table III). In 10 ng/ml TNF-α-preincubated aortic segments, the presence of 10^7 platelets/well

TABLE II. Expression of the Cytoskeleton-Associated Proteins in the Bovine Vascular Wall (Modifications Induced by Platelets)

	Normal vascular wall			Pre-inflammated vascular wall		
Protein	Control n = 18	10^5 Platelets/well n = 10	10^7 Platelets/well n = 9	Control n = 13	10^5 Platelets/well n = 10	10^7 Platelets/well n = 10
Microfibril-associated glycoprotein 4	17.1 ± 2.6	17.3 ± 2.9	25.1 ± 3.0*	11.0 ± 3.0	17.8 ± 3.6	14.5 ± 5.8
α-Actinin-associated LIM protein	6.0 ± 1.1	2.4 ± 0.6*	1.9 ± 0.2*	3.5 ± 1.1	2.3 ± 0.5	3.3 ± 0.9
α-Actin						
Isotype 1	3.9 ± 0.5	3.5 ± 0.6	3.5 ± 0.4	3.8 ± 0.6	5.5 ± 1.3	4.5 ± 1.2
Isotype 2	6.7 ± 1.3	1.6 ± 0.2*	2.0 ± 0.4*	4.2 ± 0.6	7.6 ± 1.4*	7.5 ± 1.4*
β-Actin						
Isotype 1	76.0 ± 11.5	245.9 ± 40.6*	186.4 ± 30.6*	92.5 ± 34.9	184.6 ± 73.0	322.5 ± 53.8*
Isotype 2	8.2 ± 1.5	5.3 ± 1.2	3.9 ± 0.8	2.6 ± 1.0	3.6 ± 0.8	3.3 ± 1.3
β-Tubulin	8.5 ± 1.7	3.5 ± 0.6	2.9 ± 0.5*	7.7 ± 1.4	8.7 ± 1.6	4.0 ± 1.4
Annexin						
A2	7.1 ± 1.3	7.5 ± 1.4	7.3 ± 1.1	7.9 ± 1.8	11.8 ± 5.0	16.8 ± 4.4
A4	6.5 ± 0.9	4.9 ± 1.2	4.8 ± 1.1	6.5 ± 1.7	6.4 ± 1.3	10.2 ± 2.9
A5 (isotype 1)	8.0 ± 2.0	3.3 ± 0.7*	3.3 ± 0.6*	3.2 ± 0.6	7.5 ± 2.4	3.1 ± 0.9
A5 (isotype 2)	5.5 ± 1.1	3.1 ± 0.7	2.3 ± 0.4	6.4 ± 1.3	5.1 ± 1.5	3.2 ± 0.8*
Tropomyosin β-chain						
Isotype 1	10.0 ± 2.0	2.7 ± 0.6*	0.8 ± 0.2*	5.4 ± 1.1	4.5 ± 1.6	2.3 ± 0.7*
Isotype 2	8.6 ± 1.5	6.0 ± 1.1	4.2 ± 1.0	6.3 ± 1.1	6.1 ± 1.8	6.5 ± 1.5
SM22/transgelin	11.2 ± 2.7	3.5 ± 0.9*	3.0 ± 0.5*	2.5 ± 1.0	1.5 ± 0.5	1.9 ± 0.7

AU, arbitrary units. Results are represented as mean ± SEM. The number of experiments for each experimental condition is shown in the table (n).
*$P < 0.05$ with respect to the corresponding control situation.

TABLE III. Expression of Proteins Associated With Energetic Metabolism in the Bovine Vascular Wall (Modifications Induced by Platelets)

Protein	Normal vascular wall			Pre-inflammated vascular wall		
	Control n = 18	10^5 Platelets/well n = 10	10^7 Platelets/well n = 9	Control n = 13	10^5 Platelets/well n = 10	10^7 Platelets/well n = 10
Glyceraldehyde-3-phosphate dehydrogenase	1.8 ± 0.6	2.1 ± 0.5	1.2 ± 0.1	2.0 ± 0.7	3.6 ± 0.8	6.7 ± 1.8*
Phosphoglycerate mutase	6.9 ± 0.8	5.5 ± 1.2	3.8 ± 0.6	3.6 ± 0.7	4.0 ± 0.8	2.5 ± 0.5
Mitochondrial aldehyde dehydrogenase						
Mitochondrial aldehyde dehydrogenase isotype I	7.9 ± 1.1	3.7 ± 0.7*	2.3 ± 0.3*	7.8 ± 1.8	3.2 ± 0.7	5.3 ± 1.7
Mitochondrial aldehyde dehydrogenase isotype II	7.5 ± 1.4	11.2 ± 2.9	5.5 ± 0.8	5.5 ± 0.6	3.9 ± 0.7	7.3 ± 2.0
ATP synthase β-chain	5.4 ± 0.9	4.6 ± 0.9	3.6 ± 0.6	10.4 ± 2.3	6.1 ± 1.3	8.1 ± 2.5
Glutathione-S-transferase	7.9 ± 1.2	5.4 ± 1.5	3.8 ± 0.8*	5.0 ± 2.1	4.0 ± 0.9	3.7 ± 0.7

AU, arbitrary units. Results are represented as mean ± SEM. The number of experiments for each experimental condition is shown in the table (n).
*$P < 0.05$ with respect to the corresponding control situation.

upregulated the protein expression of glyceraldehyde-3-phosphate dehydrogenase (Table III and Fig. 1). The level of expression of the other proteins identified in the pre-inflammated vascular wall were not modified by PRP (Table III).

To know more in depth the effects of PRP on the expression of the energetic metabolism-related proteins, we further determined by Western blot the protein expression of triosephosphate isomerase and fructose 1,6-bisphosphate aldolase, two glycolytic key step proteins, and ATP synthase α-chain, a protein associated with oxidative phosphorylation. In normal aortic segments, PRP failed to modify the protein expression of any of the above-mentioned proteins (Fig. 2A). However, in aortic segments submitted to a pre-inflammatory situation the protein expression of fructose 1,6-biphosphate aldolase was downregulated by platelets (Fig. 2A). The presence of PRP did not modify the protein expression of triosephosphate isomerase and ATP synthase α-chain in TNF-α-preincubated vascular segments (Fig. 2A).

The activity of fructose 1,6-biphosphate aldolase and triosephosphate isomerase was also determined in TNF-α-preincubated vascular segments. The presence of 10^7 platelets/well significatively reduced fructose 1,6-biphosphate aldolase activity without modification on the triosephosphate isomerase activity (Fig. 2B).

Finally, the pyruvate content in the bovine aortic segments was also analyzed. In the normal vascular wall the pyruvate content was not modified by the presence of 10^7 platelets/well. However, in TNF-α-preincubated aortic segments the presence of 10^7 platelets/well significatively reduced the pyruvate content (Fig. 2C).

DISCUSSION

The present study shows that in normal healthy vascular wall the presence of PRP affected the level of expression of some proteins associated with the cytoskeleton and contractile system and with the energetic metabolism. Moreover, when the vascular wall was submitted to a pre-inflammatory situation, the presence of PRP modified the expression of a lesser number of proteins associated with these processes.

It is widely recognized that both healthy and stimulated vascular wall regulates platelet activity [Bombeli et al., 1998]. In this regard, although some works have shown that in the vascular wall platelets may regulate the expression of inflammatory-associated proteins [Gawaz et al., 1998; González-Fernández et al., 1998], little is known about the effects of platelets as regulators of the expression of proteins involved in other mechanisms such as the cytoskeleton and contractile-related proteins and proteins associated with the energetic metabolism. In addition, it should be interesting to know whether platelets may modify these proteins in either healthy vascular wall and in a pre-inflammatory vascular situation, as it occurs in the cardiovascular diseases.

The first observation of the present work was that in the healthy vascular wall, PRP downexpressed a number of proteins associated with the cytoskeleton and contractile system. In this regard, in the healthy vascular wall the presence of PRP modified the protein expression of α-actinin-associated LIM protein, α-actin isotype 2, β-tubulin, tropomyosin β-chain isotype 1, and SM22/transgelin. Accordingly with our observation, Li et al. [1997] have previously demonstrated that factors released from platelets, such as platelet-derived growth factor, may inhibit the expression of smooth muscle α-actin. Moreover, Je and Sohn [2007] demonstrated that the lack of SM22/transgeling in mice decreased vascular contractile capability of the vessel. β-Tropomyosin is another protein involved in the contractile system of the vascular wall. It is interesting to note that it is known that platelets release a number of substances that may cause vasoconstriction in a damaged vascular vessel whereas may promote vasorelaxation in the healthy blood vessels [Houston et al., 1986; Raymenants et al., 1993]. Therefore, the fact that PRP downregulated these proteins associated with the contractile system in the healthy vascular wall may be in accordance with such a reduction of vascular contractility by platelets The fact that two proteins associated with the contractile system, microfibril-associated glycoprotein 4, a component of the elastic fibers, and β-actin isotype 1, were upexpressed in the healthy vascular wall by the presence of PRP may indicate a counterbalance mechanism for the downexpression of the other above mentioned contractile proteins.

In the present work, when a pre-inflammatory state was induced in the vascular wall, the presence of PRP also modulated the expression of contractile-related proteins but in lesser number than in the healthy aortic segments. Moreover, proteins like microfibril-associated glycoprotein 4, β-tubulin and SM22/transgelin that they were down or upexpressed by platelets in the healthy aortic segments, in TNF-α-preincubated aortic segments these proteins

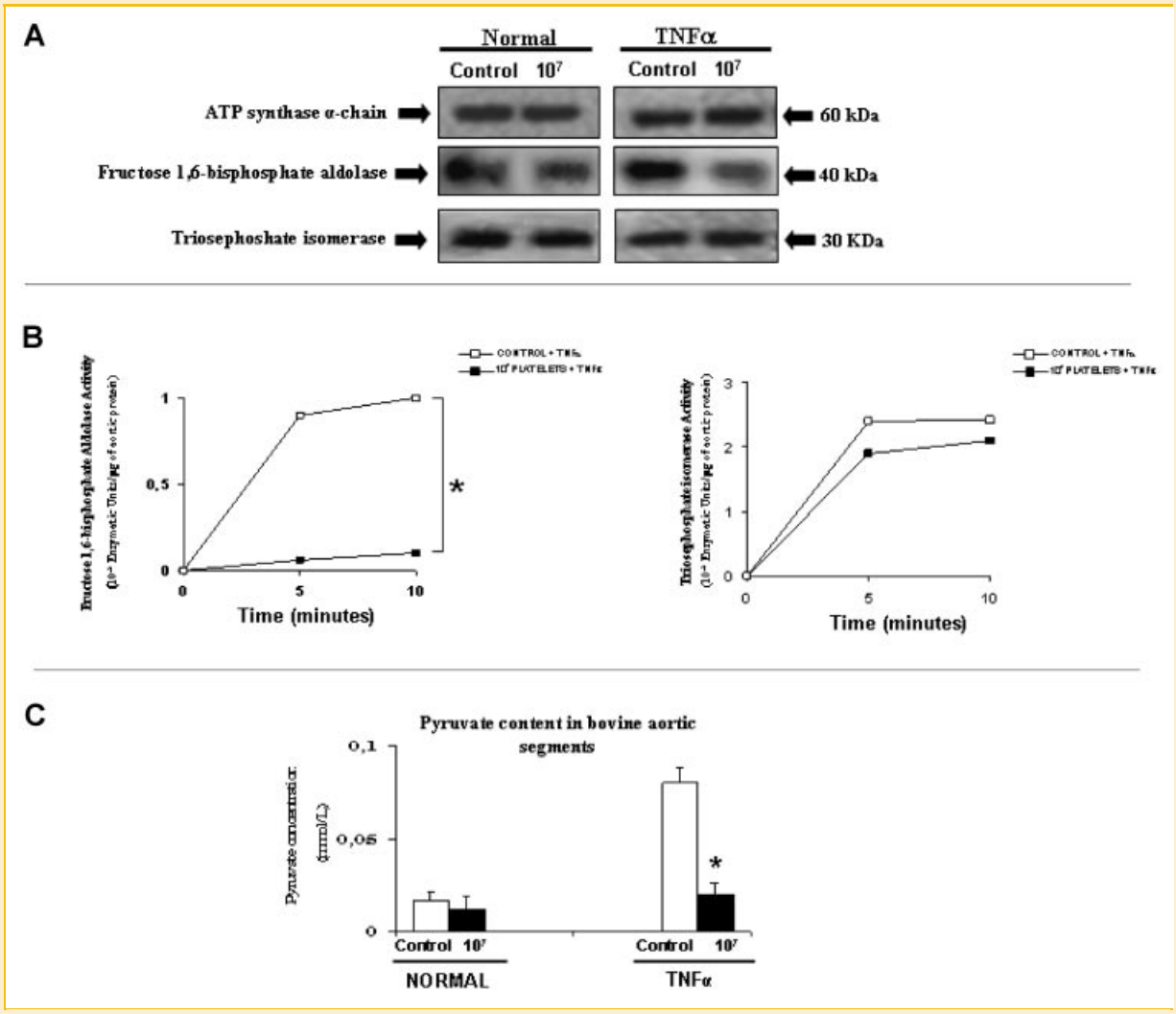

Fig. 2. A: Representative Western blots showing the effects of human platelets (10^7 platelets/well) on the expression of ATP synthase α-chain, fructose 1,6-bisphosphate aldolase and triosephosphate isomerase in control and 10 μg/ml TNFα-preincubated aortic segments. B: Scatter plots showing fructose 1,6-bisphosphate aldolase (left) and triosephosphate isomerase (right) activities in the pre-inflammated vascular wall. Fructose 1,6-bisphosphate aldolase and triosephosphate isomerase activity was recorded during 10 min. Results are represented as 10^{-5} enzymatic units/μg of aortic protein. $^*P < 0.05$ with respect to the corresponding control situation. C: Bar graphs showing the effect of platelet (10^7 platelets/well) on the pyruvate content in control bovine aortic segments and 10 μg/ml TNFα-preincubated bovine aortic segments. Results are mean ± SEM. $^*P < 0.05$ with respect to the corresponding control situation.

were not modified by the presence of platelets. Evenmore, proteins like β-actin isotype 1 and tropomyosin β chain isotype 1 that they were modified by both 10^5 and 10^7 platelets/well in the healthy aortic segments, in TNF-α-preincubated aortic segments they were only modified by 10^7 platelets/well. Taken together, these results may apparently suggest a dual molecular effect of platelets on the vascular contractile system depending of the inflammatory situation of the vascular wall. It is may be in accordance with the widely reported and above mentioned observation that platelets induce vasodilatation on the healthy but vasoconstriction in the sick vascular wall.

In our study, the presence of PRP downexpressed annexin A5 isotype 1 in the healthy vascular wall and 10^7 platelets/well reduced annexin A5 isotype 2 in TNF-α-preincubated aortic segments. Annexins are involved in the organization of membrane-associated cytoskeleton and has been also identified as important regulators of the haemostatic balance [Gerke, 1992; Ling et al., 2004]. Numerous functions have described for annexin A5 in in vitro studies although it is in vivo role still remains unclear. In this regard, annexin A5 has been proposed as a potencial tracer for imaging apoptosis in atherosclerotic plaques. Indeed, a higher annexin A5 accumulation in grade IV atheroma than in other more stable lesions [Zhao et al., 2007].

Glucose metabolism seems to be an important determinant of vascular reactivity [Lund et al., 2000]. In our study, an increased expression of glyceraldehyde-3-phosphate dehydrogenase but a reduced expression and even activity of 1–6 biphosphate aldolase, a key-step glycolytic enzyme [Villar-Palasi and Larner, 1970], were observed when TNF-α-preincubated aortic segments were coincubated with PRP.

However, another key-step glycolytic enzyme, triosephosphate isomerase [Villar-Palasi and Larner, 1970], did not change its expression and activity in TNF-α-preincubated aortic segments coincubated with PRP. The increased glyceraldehyde-3-phosphate dehydrogenase expression observed in the pre-inflammed vascular wall coincubated with PRP could reflect a compensatory mechanism within the glycolytic pathway due to the reduction of both protein expression and activity of fructose 1,6-biphosphate aldolase. In this regard, the vascular content of pyruvate, the end-product of the glycolytic pathway, was reduced by PRP in the TNF-α-preincubated aortic segments suggesting a diminished glycolytic activity.

It is remarkable that glyceraldehyde-3-phosphate dehydrogenase has also the ability to display other activities further to its glycolytic involvement [Sirover, 1999]. Upexpression of glyceraldehyde-3-phosphate dehydrogenase has been involved in smooth muscle cell proliferation [Ranganna et al., 1995]. In our results, glyceraldehyde-3-phosphate dehydrogenase was upexpressed only when the vascular wall was submitted to a pre-inflammatory situation. Previous studies have suggested that glyceraldehyde-3-phosphate dehydrogenase was upexpressed by platelet-derived growth factor BB [Ranganna and Yatsu, 1997]. Therefore, when the vascular wall is stimulated, as it occurs under the pre-inflammatory state, platelet secretion of growth factors, including platelet derived growth factor BB, may favor glyceraldehyde-3-phosphate dehydrogenase upexpression.

Mitochondrial aldehyde deshydrogenase is an enzyme responsible for the oxidation of aldehydes to carboxylic acids. Mitochondrial aldehyde deshydrogenase also catalyzes the formation of 1,2-glycerol dinitrate and nitrite from nitroglycerin within mitochondria leading cGMP-dependent vasorelaxation. Interestingly, glutathione-S-transferase also potentiated platelet inhibition by nitroglycerin suggesting that glutathione-S-transferase may be involved in nitroglycerin transformation into nitric oxide [Miller et al., 1989]. In the coincubation experiments, platelets reduced the protein expression of both mitochondrial aldehyde dehydrogenase isotype 1 and glutathione-S-transferase in the healthy vascular wall with may suggest that platelets could modulate the vasodilating response to nitrovasodilators. However, in our knowledgment there are not published data about the possible effect of platelets on the vasodilating response to nitrovasodilators.

An important function of glutathione-S-tranferase is its protective effect on the degradation of biological membranes through glutathione SH-dependent reduction of nonspecific peroxidized phospholipids [Vaziri et al., 2003; Ceballos-Picot et al., 2004]. The reduced expression of glutathione-S-transferase in the healthy vascular wall by PRP may be associated with a reduced vascular defense against oxidants. Therefore, platelets by themselves may try to promote their activation fighting against the anti-thrombotic properties of the healthy vascular wall.

A limitation of the present study could be the species heterogeneity since human platelets were coincubated with bovine aortic vascular segments. Bovine platelets were not available to be used since once blood sample is obtained PRP has to be immediately isolated to minimize the activation of platelets. The distance between the abattoirs to the experimental laboratory did unable to use bovine platelets. However, it is important to remark that the presence of platelets failed to modify the expression of other proteins in the bovine vascular wall which support the specificity of the observed changes.

In pathophysiological conditions platelets may roll and physically interact with the endothelium. However, in the present experimental design, platelets and the vascular wall was separated by a membrane although substances released from each of them may affect one to the other. The question then raised is how inactivated platelets may regulate the expression of proteins in the vascular wall? An explanation about it is that resting platelets may release a number of vasoactive factors that probably may affect protein synthesis in the vascular wall and even in other blood cells [Zhou et al., 1995; Xu et al., 1997; Raiden et al., 2003]. In this regard, a limitation of the study, which it is shared for to all the studies performed using isolated PRP, is that during the centrifugation processes to obtain PRP, a certain degree of platelet activation may occur which may favor the release of such substances from the platelets. That is also why the experiments were performed using PRP instead of isolated platelets. Since it is a more physiological approach because the processes to isolate platelets may increase more the spontaneous activation of platelets since to obtain isolated platelets requires additional centrifugations.

It is also plausible that some of the here reported changes attributed to platelets may be influenced by the plasma contained in PRP. However, we cannot discard that elements contained in the plasma the platelet effect on the vascular wall. However, control experiments were performed with the same amount of PPP than the final volume of PRP + PPP contained in 10^5 platelets/well since the greater amount of plasma was present in these experiments than these with 10^7 platelets/well. This fact minimized the possible effect of plasma in the here reported protein changes by PRP.

In summary, the main finding of the present study was that in the control vascular wall platelets by themselves affected the expression of a number of proteins associated with contractile system and the energetic metabolism. However, under vascular pre-inflammatory state, platelets modified the expression of a lesser number of proteins associated with contractile system and energetic metabolism. Therefore, the present study provides new evidences about the main implication of platelets in the regulation of the expression of vascular proteins particularly under healthy and inflammatory vascular conditions which they may have importance in the setting of ischemic processes.

ACKNOWLEDGMENTS

This work has been supported by Fondo de Investigaciones de la Seguridad Social (Redes temáticas de Cooperación Red Heracles RD06/0009/010) and by Fondo de Investigaciones de la Seguridad Social (FIS04/1205). Petra J Mateos-Cáceres is staff of Fundación para la Investigación Biomédica del Hospital Clínico San Carlos. Jose Javier Zamorano-León and Javier Modrego are staff of Red Heracles. The authors thank Begoña Larrea for secretarial assistance.

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