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Proteomics Analysis of Morphogenic Changes of Human Umbilical Vein Endothelial Cells Induced by a Phorbol-Ester Mimicking Angiogenesis

Baudin Bruno*[1, 2], Ramani David[1], Bosselut Nelly[1], Marcelo Paulo[3], Lecornet Hélène[1], Margoline Gary[1], Vinh Joëlle[3], Vaubourdolle Michel[1]

[1]Biochimie A - Pôle Biologie Médicale et Pathologie - Hôpitaux Universitaires Est Parisien (APHP), Site Saint-Antoine - 184 Rue du Faubourg Saint-Antoine 75571 Paris cedex, France; [2] EA 4530 - UFR Pharmacie Université Paris-Sud, 5 rue Jean-Baptiste-Clément 92296 Châtenay-Malabry cedex, France; [3] CNRS-USR 3149, ESPCI Paris Tech, 10 rue Vauquelin, 75231 Paris cedex 05, France.

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ABSTRACT

Phorbol 12-myristate 13-acetate (PMA) can induce proliferation and migration of endothelial cells, mimicking vessel formation. We analysed by two-dimensional electrophoresis and MALDI-TOF/TOF the effects of PMA on cultured Human Umbilical Vein Endothelial Cells (HUVECs) to further investigate the complex mechanisms related to protein kinase C activation in this angiogenesis model. At 1 µg/ml for 24 hours, PMA induced transition of HUVECs from quiescent type into the proliferative-migrating phenotype. After 2D gel analysis, 15 differences were detected between PMA-treated samples and controls, including 8 increased proteins and 7 decreased proteins. The three main proteins identified by mass spectrometry and increased after PMA are directly involved in cell stress (α-glucosidase, heat-shock protein 70, and 150 kDa oxygen-regulated protein). Four other proteins varied in function of time, two increasing after PMA (heat shock protein 90β, protein-disulfide isomerase A3), and two other decreasing after this treatment (glucose-related protein 75, cathepsin B). These four proteins are involved in protein folding, apoptosis or tumour dissemination. Our data show that phorbol esters modify a number of proteins involved in multiple and intricate pathways for promoting a phenotype ensuring cell survival and cell migration for new vessels formation.

Keywords: Angiogenesis; endothelial cells; oxygen-regulated protein 150; phorbol esters; protein kinase C; proteomics.

Abbreviations

HUVECs, human umbilical vein endothelial cells; **PMA**, phorbol 12-myristate 13-acetate; **PKC**, protein kinase C; **HSP-70**, heat-shock protein 70; **HSP-90β**, heat-shock protein 90 beta; **GRP-75**, glucose-related protein 75; **ORP-150**, oxygen-regulated protein 150; **PDI-A3**, protein-disulfide isomerase A3; **VEGF**, Vascular endothelial growth factor.

1. Introduction

Angiogenesis is the formation of new capillaries from a pre-existing capillary network and is implicated in many physiological or pathological processes, particularly carcinogenesis and diabetic retinopathy. Both physiological and pathological angiogenesis are regulated by a tight balance between activators, such as "vascular endothelial growth factors" (VEGFs) and angiopoietins, and inhibitors, such as thrombospondin 1 and endostatin [1, 2]. Endothelial cells (ECs) play a key role in both mechanisms of angiogenesis, i.e. "sprouting" with EC migration, and "intussuceptive angiogenesis" with ramification of pre-existing blood vessels [3]. These two processes need pericytes recruitment and secretion of an extra-cellular matrix. Phorbol-esters, in particular phorbol 12-myristate 13-acetate (PMA), are protein kinase C (PKC) activators known to promote blood vessel formation [4, 5]. PMA is sufficient for the induction of angiogenesis and PKC is necessary for this process [6-8]. Activation of PKC by PMA has a variety of effects on cultured ECs, in-

***Corresponding author:** Bruno Baudin. Service de Biochimie A, Hôpital Saint-Antoine, 184 rue du Faubourg Saint-Antoine 75571 Paris cedex, France; Tel : 33 1 49 28 20 13; Fax : 33 1 49 28 20 77; E-mail Address: bruno.baudin@sat.aphp.fr

cluding induction of migration, proliferation and vessel formation [9-11], and essentially via induction of VEGF [12]. Since treatment of EC with PMA is able to mimic the *in vivo* angiogenic process, it has provided a valuable tool for the investigation of signalling pathways involved in angiogenesis [13]. In particular, in Human Umbilical Vein Endothelial Cells (HUVECs), PMA-activated PKC activates both Raf/MEK/ERK and MAPK signalling pathways [14-16]. ERK phosphorylates the protein complex binding the Egr-1 gene leading to the induction of gene expression. ERK is also able to activate *c-jun* forming the AP-1 complex. Both AP-1 and Egr-1 transcription factors bind their target sequence to promote gene expression. To date, at least 12 distinct members of the PKC superfamily have been identified in mammalian cells [17], but mainly PKC isoforms α and δ have been implicated in the regulation of angiogenesis [18, 19]. For example, down regulation of PKCα in HUVECs by antisense oligonucleotides inhibits vessel formation [20]. Other studies have shown that sphingosine kinase is activated by PKC leading to the formation of sphingosine 1-phosphate with angiogenic properties [21-23]. PMA is also effective in inducing morphogenic changes in HUVECs by activating both PI_3 kinase and Akt/protein kinase D (PKD) pathways. Moreover PKD is phosphorylated in response to PMA and was reported to directly stimulate ERK activation in ECs in response to VEGF [7, 24]. PMA and VEGF can induce CREB-mediated COX-2 expression through a PKCα dependent pathway in human endothelium [25]. These studies reveal the complexities of the pathways governing vessel formation. PMA was also shown to promote the adhesion of endothelial progenitor cells to vascular endothelium and extracellular matrix without particular toxicity and negligible impact on cell survival [26].

In our study, we analysed the effects of PMA on primary cultures of HUVECs to further investigate these complex mechanisms, using two-dimensional electrophoresis (2-DE) coupled with MALDI-TOF/TOF mass spectrometry for protein identification.

2. Material and Methods

2.1. *HUVEC culture*

This protocol was previously described in details [27]; only primary cultures were used for this study and they were obtained by mixing ECs from two umbilical cords. As observed by phase-contrast optic microscopy, all the cultures reached cell confluence 7 days after seeding in 35mm Petri dishes.

2.2. *PMA treatment and sample preparation*

At this stage, the cultures were washed in PBS (3 times) and half of them were incubated with PMA (Sigma, Saint-Quentin Fallavier, France) at a concentration between 0.5 and 2 µg/ml, the other half with culture medium (controls), for 14 to 48 hours at 37°C in 5% CO_2-95% air atmosphere saturated with water. For incubation with PMA, the foetal calf serum used for cell growth was replaced with Ultroser G (Biosepra, Cergy-Pontoise, France) at 1% in culture medium; PMA was diluted in this medium. After incubation, culture medium was eliminate; the remaining adherent cells were washed in PBS (3 times) and mechanically scrapped in 4% Chaps, 2.3% dithiothreitol (DTT), 5mM N-ethylmaleimide and 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride (detergent, disulfide bond reducer and protease inhibitors, respectively). Protein concentration was determined after this stage, using 2D Quant Kit (General Electric Healthcare, Vélizy-Villacoublay, France) and bovine serum albumin (Sigma) as a standard. Finally, the samples with similar protein contents were treated with cold acetone (4/1 v/v) for protein precipitation (2 hours at - 20°C); after centrifugation (1500g, 15 min at + 4°C), the pellet was dissolved in buffer for 2-DE analysis performed as previously described [28].

2.3. *2-DE, statistical analysis and peptide preparation*

All these procedures were detailed in our previous works on proteomics of HUVECs [28, 29]. In this study, for the first dimension, we used IPG-Phor and pH 4-7 non linear IPG 18cm strips (GE Healthcare), and for the second dimension, home made 10% SDS-PAGE with Bio-Rad (Marnes-la-Coquette, France) materials. The gels were coloured with mass spectrometry compatible silver nitrate or colloidal Blue Coomassie as it applied, then scanned with ImageScanner and studied with ImageMaster 2D Platinium™ (GE Healthcare). For the first set of experiments (1 µg/ml PMA/control), the three 2D gels from PMA-treated cells were compared to the three gels from non-treated cells (control) after studying the reproducibility between the three gels of each class using bio-informatics set in the software. For the second set of experiments (kinetic study), six gels were performed, two for each time and condition (1 µg/ml PMA/Control). Statistical comparisons were performed using Wilcoxon tests ($p < 0.05$ was considered significant); they were based on normalized volumes of the spots.

For both sets of experiments, spots corresponding to statistically significant variations were manually excised and treated for peptide preparation as described in detail before, particularly using trypsin, DTT for disulfide bridge reduction, and iodoacetamide for thiol alkylation [30].

2.4. *Protein identification by MALDI-TOF/TOF*

MALDI-TOF/TOF-MS experiments were carried out using a tandem mass spectrometer 4800 MALDI TOF/TOF™ Analyser (Applera Applied Biosystems, Framingham, MA). MS analyses were performed in positive ion reflectron mode, with an accelerating voltage of 20kV; 0.3 µl of the samples were deposited with 0.6 µl of α-cyano-4-hydroxycinnamic acid matrix (4 mg/ml) dissolved in a 60% acetonitrile, 40% H_2O (v/v), 0.1% trifluoroacetic acid and 10mM ammonium citrate solution. For subsequent data processing, the GPS

Explorer™ Workstation (Applied Biosystems) was used before the identification with MASCOT software (http://ww.matrixscience.com). An external calibration was achieved with a standard peptide mix (Proteomix Peptide mix4, LaserBioLabs, Sophia-Antipolis, France) containing: bradykinin fragment 1-5 (573.3150 Da), human angiotensin II (1046.5424 Da), neurotensin (1672.9176 Da), ACTH fragment 18-39 (2464.1989) and oxidized insulin B chain (3494.6514 Da). The MASCOT program was used with the following parameters: human species, 2 missed cleavages by trypsin, monoisotopic peptide masses, mass deviation of 100ppm (MS) and 0.15 Da (MS/MS). Oxidation of methionine and carbamidomethylation of cysteine were taken into consideration. All the proteins identified should have a protein score (exponential score) greater than 60 (Confidence Interval with this software) and individual ion score greater than 40 was set as the threshold for acceptance (MASCOT linear score) with at least two different identified peptides.

3. Results

Used at 1 µg/ml PMA induced morphogenic effects in HUVECs with elongation of the cells but without new blood vessels promotion (Figure 1). At 0.5 µg/ml these effects were lower, whereas at much lower concentration (e.g. 0.1 µg/ml), PMA failed to induce this particular phenotype. When used at 2 µg/ml PMA induced toxic effects with cell disruption and detachment, but, conversely, in some areas tube-like structure could be observed (not shown). As at this concentration PMA exerted ambiguous effects, we chose the 1 µg/ml concentration for the following studies. Then, we performed a kinetic study with 1 µg/ml PMA for 14h, 24h or 48h of incubation: at 14h of incubation the elongation of the cells was already perceptible, but less intensive than at 24h or 48h. The most intense effects, and sometimes with tube-like structure formation, were shown after 48h, but some cells seemed to suffer at this longer time. Whatever the tested concentration or time, we could not observe fibroblast-like structures that may come from the differentiation of ECs into smooth muscle cells, since HUVECs retain this potential in vitro when deprived of fibroblast growth factor [31]. Indeed PMA mimics VEGF effects on HUVECs inducing transition from the quiescent into the proliferating-migrative phenotype.

In the first set of 2-DE experiments, three different cultures of HUVECs were performed in both conditions, 1 µg/ml PMA-treated cells versus non-treated cells (controls) for 24h of incubation. Two-D gels were compared with software and bio-informatics. The mean number of spots was 1176 ± 15 for the control group and 1247 ± 104 for the PMA group, a difference not statistically significant. The coefficient of correlation for matched spots was 0.825 between gels from control group, and 0.81 between gels from PMA group showing identical reproducibility for both groups. Moreover, between PMA-treated and control 2D gels the coefficient of correlation was 0.871 (the regression for matching spots was $y = 0.98 x + 0.001$) showing that most of proteins were not modified after PMA treatment. When the bio-informatics analysis was restricted to matched spots, 15 differences were detected, including 8 proteins with enhanced level and 7 proteins with a decrease level after PMA treatment. All the spots showing increased levels of proteins in PMA group were analysed by MALDI-TOF/TOF with peptide sequencing for protein identification: 6 proteins were identified, identically in one gel from each group when possible (Table 1). As keratins and cytokeratins could have contaminated the peptide preparation, they were not further taken into consideration. Albumin (in two forms) could have also con-

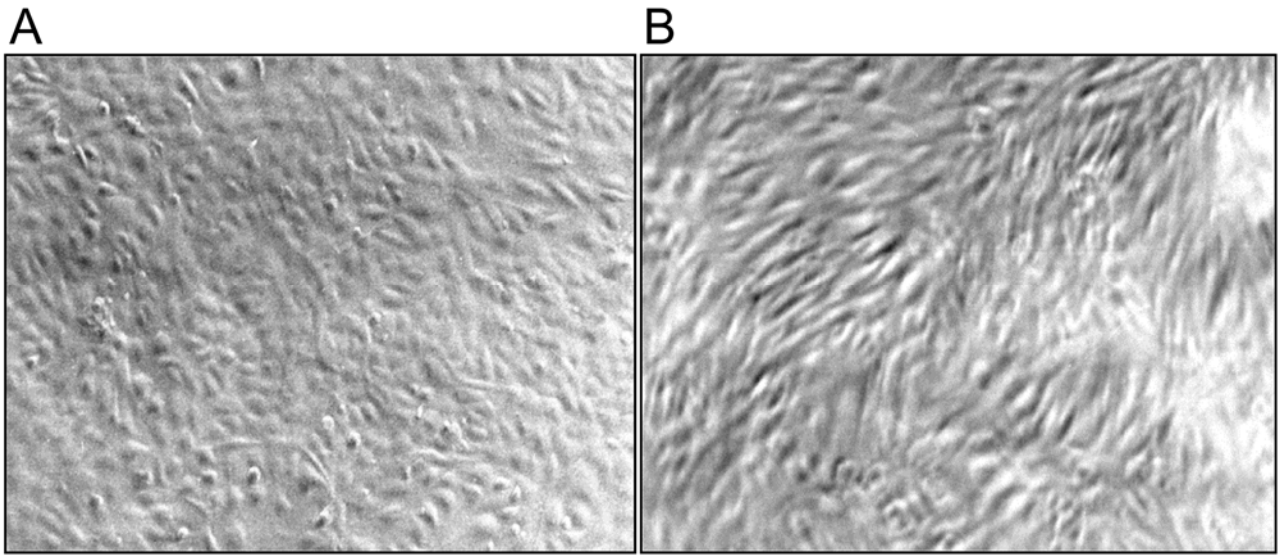

Figure 1. Morphogenic effect of PMA on HUVECs. A- Control (HUVECs at confluency). B- HUVECs treated with 1 µg/ml PMA for 24 hours. In these conditions, HUVECs presented a more elongated morphology than control cells.

Table 1. Proteins identified by MALDI-TOF/TOF differentially expressed in PMA-treated and non treated cell samples (PMA/Control); first set of experiments.

Identification	SwissProt accession n°	Protein score	Number of used peptides	Theoretical pI/Mr (kDa)	Main function and location	Spot number
Neutral alpha-glucosidase AB precursor	Q14697	497/497	33/35	5.74/106.8	Glycolysis, cytosol	144
Stress-70 protein, mitochondrial precursor	P38646	660/595	31/29	5.87/73.6	Chaperone, mitochondria	238
150 kDa Oxygen-regulated protein precursor	Q9Y4L1	174/68	13/14	5.16/111.3	Chaperone, endoplasmic reticulum	609
Isocitrate dehydrogenase (NADP)	O75874	68/71	9/7	6.53/46.6	Malic cycle, cytosol	609
Human serum albumin precursor	P02768	107/107	11/14	5.92/69.3	Plasma protein	1263
Bovine serum albumin precursor	P02769	874/822	34/33	5.82/69.2	Plasma protein	1263

taminated HUVEC preparation since foetal calf serum contains this protein. Nevertheless this particular cell type was shown to up take albumin in a more or less specific manner [32]. The three main interesting identifications were α-glucosidase, 70 kDa heat-shock protein (HSP-70) and 150 kDa oxygen-regulated protein (ORP-150), all enhanced after PMA-treatment with statistically significant increments ($p<0.05$) (Figure 2).

In a second set of 2-DE experiments, we compared 3 times of incubation (14h, 24h and 48h) with or without 1 µg/ml PMA; two 2D gels we realized for each time and condition, thus six gels in total. Gels from PMA-treated cells were compared to their controls. Other variations than in the first set of experiments were shown, but we only retained the variations in spots well matched on at least five gels. Finally the statistical significance was reached for 10 spots: 6 with enhanced protein levels in PMA-treated cells, 4 with decreased protein level in these cells compared to controls. After MALDI-TOF/TOF analysis, 4 proteins were identified : (i) HSP-90β and protein-disulfide isomerase A3 (PDI-A3), both increased in PMA-treated cells in function of time; (ii) 75 kDa glucose related protein (GRP-75) and cathepsin B, both decreased in these conditions (Table 2). More precisely, cathepsin B was expressed at low level in PMA-treated cells but was progressively more expressed in function of time reaching cathepsin B level in control cells. GRP-75 was always expressed at high level in control cells in comparison of PMA-treated cells. Conversely, HSP-90β and PDI-A3 were always expressed at high level in PMA-treated cells in comparison of controls (Figure 3).

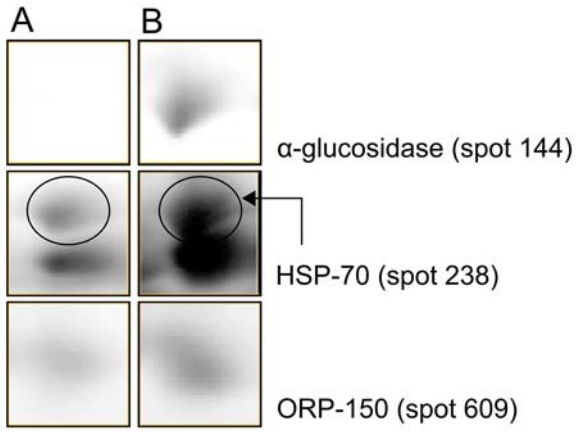

Figure 2. Protein identifications from the first set of experiments. Magnification of the 3 spots with interesting identified proteins differentially expressed in HUVECs treated with 1 µg/ml PMA for 24 hours (B) compared with control cells (not treated by PMA)(A).

4. Discussion

In our study, PMA has shown a toxic effect from 2 µg/ml even when this concentration showed more drastic morphogenic effect mimicking angiogenesis. In many studies on HUVECs, PMA was used in far more little concentrations, typically between 0.01 and 0.1 µg/ml, and often for incubations under 24 hours [7, 14, 33]. For the proteomics studies, we chose a concentration of PMA leading to a typical morphogenic effect without toxicity, and for incubations between 14 and 48 hours, Ultroser G, used as a supplement medium, was added in place of foetal calf serum. At 48 hours the morphogenic modifications were always higher than at 24 or 14 hours. Used at higher concentration, PMA was toxic for HUVECs.

In the first proteomics study, PMA was used at 1 µg/ml for 24 hours; on 2D gels, after bio-informatics analysis, 8 spots showed an enhanced level and 7 a decreased level after PMA treatment; all these modifications were statistically significant. Three interesting proteins were identified by mass spectrometry using peptide mass fingerprinting and peptide fragmentation, all enhanced after incubation of the cells with

Table 2. Proteins identified by MALDI-TOF/TOF differentially expressed in function of time in PMA-treated and non treated cell samples (PMA/Control); second set of experiments (kinetic study).

Identification	SwissProt accession n°	Protein score	Number of used peptides	Theoretical pI/Mr (kDa)	Main function and location	Spot number
Glucose regulated protein-75	P48721	68	19	5.87/73.6	Chaperone, cytosol/mitochondria	1297
Protein disulfide isomerase A3	P11598	412	37	5.88/56.9	Chaperone, Endoplasmic reticulum	1438
Heat shock protein 90 beta	P34058	185	18	4.97/83.2	Chaperone, cytosol	1726
Cathepsin B	P00787	55	3	5.88/37.7	Protease, lysosome	2783

PMA, i.e. α-glucosidase, HSP-70 and ORP-150. α-Glucosidase is a glycolytic enzyme able to liberate glucose from oligosides and polyosides, in particular the α-1,4 bound in maltose and in fragments from shark or glycogen, also the α-1,6 bound of the last glucose at glycogen branching that enables the complete hydrolysis of stock glycogen in cells. The increase in α-glucosidase after PMA treatment could be an adaptation to a defect in energy, since glucose can enhance ATP production as well as in aerobic as in anaerobic conditions. HSP-70 is a mitochondrial protein with enhanced expression normally related to a response to a stress, as well as heat shock, hypoxia, deprivation in glucose, and oxidative stress [34, 35]. This heat shock protein acts as a chaperone for a number of proteins with various functions but essential for cell survival [36]. Moreover, VEGF was shown to increase the expression of many chaperones and HSPs in HUVECs, in particular the HSPs-70 p5 and p8, in parallel of the over expression of structural proteins related to cell migration such as myosin, actin and α-tubulin [37]. ORP-150 is also a chaperone but located in endoplasmic reticulum (ER); it shares 91 % of homology with glucose-related protein 170 (GRP-170) and contains ATP-binding motif. ORP-150 expression is induced by hypoxia, 2-deoxyglucose, osmotic shock and tunikamycin [38, 39]. Its chaperone activity seems specific for vascular endothelial growth factor (VEGF) that exerts pro-angiogenic and anti-apoptotic properties [40]. In particular, ORP-150 allows VEGF secretion from ER in response to hypoxia. Moreover, when ORP-150 synthesis is inhibited, VEGF is restricted to ER canal [41]. In a number of cell types, ORP-150 appears as essential for cell survival, in particular when cells are deprived in ATP or its sources such as oxygen and glucose.

In the kinetic proteomics study, four other proteins were identified, two were enhanced in PMA-treated cells (HSP-90 β and PDI-A3), and two were decreased in PMA-treated cells (GRP-75 and cathepsin B), all differentially expressed in function of time. Unfortunately, the 3 proteins identified in the first study were not further identified in the kinectic study because the corresponding spots were not found in at least 5 gels for statistical comparison. But, the 4 proteins identified in the second study have also interesting functions. HSP-90 is a chaperone playing important functions in protein maturation, in particular for proteins controlling transcription and intercellular signalling. It also exerts anti-apoptotic properties and could be regulated by mitogenic agents. For example, PMA was shown to induce HSP-90β expression in Jurkat T lymphocytes [42]. Moreover, VEGF can induce the association between HSP-90 and Akt leading to both the activation and the over expression of NO-synthase in endothelial cells [43]. PDI-A3 (or Erp57) is a ubiquitous protein expressed in endoplasmic reticulum and belongs to the large family of the PDIs indispensable for protein folding by disulfide bridge isomerisation. In endoplasmic reticulum, Erp57 is specialized for the folding of glycoproteins, in particular when complexed to chaperones such as calnexin and calreticulin; its expression is enhanced by stress stimuli [44]. GRP-75 (or mortalin), is a chaperone belonging to the HSP-70 family, in particular for the transport of proteins into the mitochondria; it could be anti-apoptotic and is over expressed in a number of cancer cell lines [45, 46]. Cathepsin B is a ubiquitous lysosomal cysteine-protease often incriminated in cancer, in particular for tumour dissemination after degradation of the extra-cellular matrix. The relation between this protease and angiogenesis is ambiguous as sometimes it was enhanced in the angiogenic phenotype of endothelial cells. For example, in a study on HUVECs, the elevation of cathepsin B activity was associated to the formation of tubular structures such as new vessels, and in other studies cathepsin B was associated to angiogenesis inhibition with the over expression of endostatin [47-49].

5. Concluding Remarks

Our data show that a phorbol ester induces a number of protein expressions involved in multiple and intricate pathways for promoting a phenotype ensuring cell survival of endothelial cells and cell migration for new vessel formation.

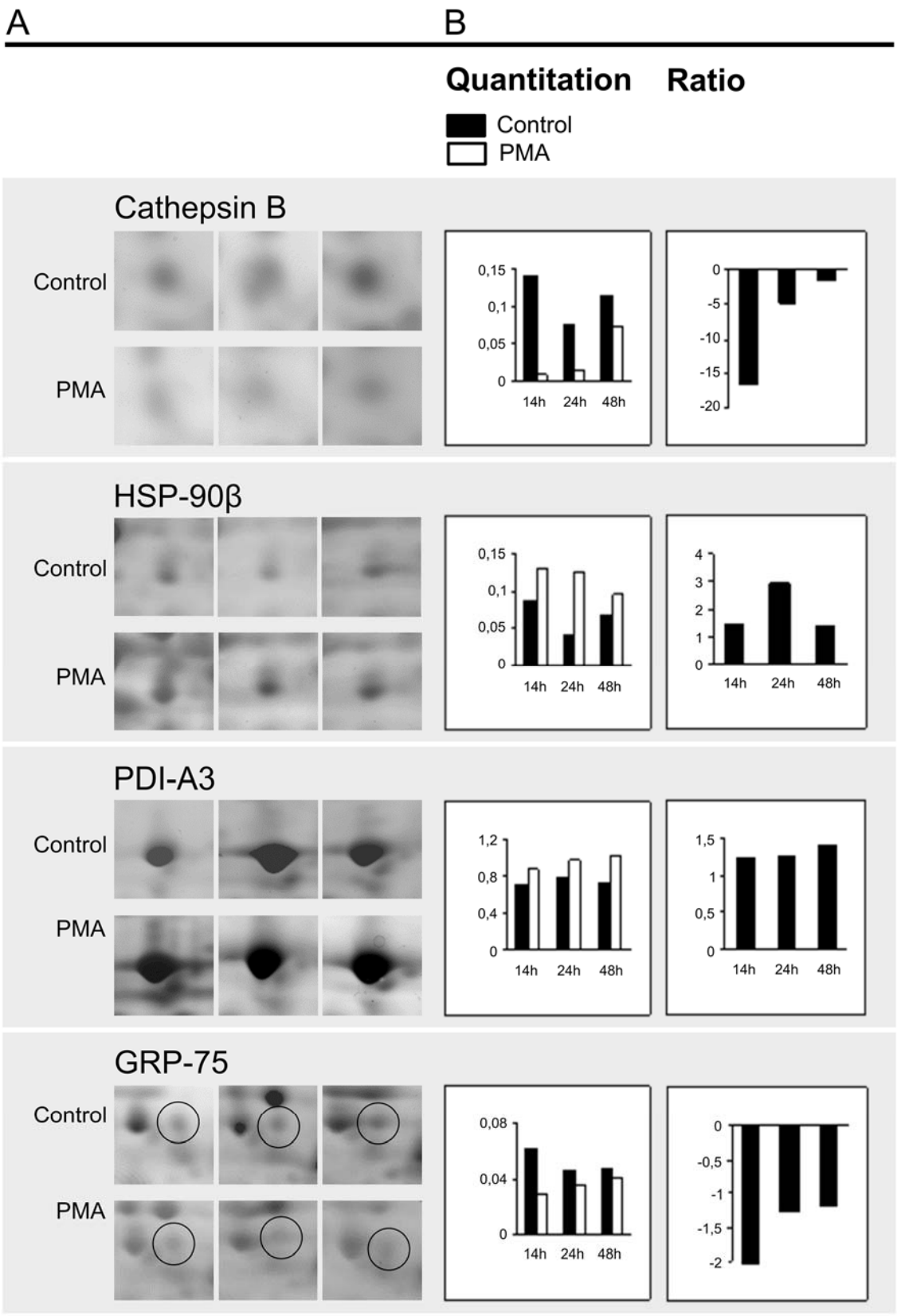

Figure 3. Protein identifications from the second set of experiments. A- Magnification of the 4 spots with interesting identified proteins differentially expressed in function of time in HUVECs treated with 1 µg/ml PMA compared with control cells (not treated by PMA). B- Histograms showing significant differences for these proteins with either enhanced or decreased levels after PMA-treatment ($p < 0.05$).

Competing interests

The authors declare no conflicts of interest for this study.

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