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Running title: *Vessel destabilization in diabetic retinopathy*

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Abstract

Aims: Diabetic retinopathy (DR) is characterized by early dropout of capillary pericytes, leading to loss of control on endothelial proliferation and, subsequently, angiogenesis. We have demonstrated that extracellular vesicles (EV) derived from mesenchymal stem cells (MSC) maintained in diabetic-like conditions may play a role in vessel destabilization, thus contributing to angiogenesis through paracrine signalling. In particular, a role for MMP-2 was described. This study was aimed at further investigating the molecular mechanisms of EV-induced vessel destabilization.

Methods: We evaluated miR-126 expression, the subsequent HIF-1α and VEGF modulation, Ang-2 and PDGF signalling pathways, in human retinal pericytes (HRP) after exposure to MSC-derived EV obtained in diabetic-like conditions (high glucose and/or hypoxia).

Results: HRP express miR-126 and this expression is down-regulated in intermittent high glucose. MSC-derived EV obtained in hyperglycaemic/hypoxic conditions down-regulate miR-126 expression in pericytes leading to increased expression of angiogenic molecules, such as VEGF and HIF-1α. No modulation of Ang-2 and PDGF signalling pathways in pericytes was observed following EV exposure.

Conclusions: HRP express miR-126 and this expression is down-regulated in diabetic-like conditions. Exposure of HRP to EV obtained in diabetic-like conditions is able to decrease miR-126 expression, consistently with previous observations of its involvement in DR and providing further insights into the role of EV in vessel destabilization. In contrast, PDGF and Ang-2 signalling pathways do not seem to be involved in these mechanisms.

Keywords: extracellular vesicles, mesenchymal stem cells, pericytes, diabetic retinopathy, angiogenesis, miR-126

Introduction

Diabetic retinopathy (DR), a sight-threatening complication of diabetes, is characterized by early loss of capillary pericytes, which, together with thickening of the basement membrane, may lead to loss of control on endothelial proliferation and, subsequently, angiogenesis [1]. Therefore, pericytes and their interactions with endothelial cells (EC) in the vessel wall play a key role in the regulation of vessel formation, stabilization and remodelling [1]. On the one hand, EC act as mediators of signalling from the blood, and on the other pericytes receive, transform and pass to the endothelium, signals from the surrounding tissues [2].

Transfer of genetic information by extracellular vesicles (EV) [3-6], has come into focus as an important modality of paracrine signalling. EV are released by several cell types in physiological and pathological conditions [7] and, as they contain biologically active molecules [7-9], may regulate cell migration, proliferation and differentiation, as well as angiogenesis [7-10]. Moreover, they have been described as potential biomarkers for metabolic diseases, such as type 2 diabetes [7]. In fact, there is evidence for increased circulating EV in diabetic animals [7] and in type 2 diabetic subjects [11].

Mesenchymal stem cells (MSC) are ubiquitous pluripotent stem cells, present also in human eye tissues [12] and known to release a high number of EV [9]. It has been hypothesized that MSC can be isolated from all vascularized organs and that perivascular cells such as pericytes, which have similar properties and share several surface markers with MSC, may be their possible source [13-14].

We have recently shown that MSC-derived EV are able to enter pericytes, causing detachment from their substrate and migration, stimulate angiogenesis *in vitro*, and that they may contribute to perturb the blood-retinal barrier permeability [2]. These effects were mediated by an increase in matrix metalloproteinase-2 (MMP-2) and exacerbated if MSC had been previously cultured in conditions (high glucose and/or hypoxia) mimicking the diabetic microvascular milieu. [2]. Therefore we hypothesized that diabetic-like conditions may influence vessel stabilization during angiogenesis through EV paracrine signalling. However, further investigations of the molecular mechanisms involved in these findings were needed.

MicroRNAs (miR) are short RNA sequences, acting as gene modulators and playing key roles in several biological processes, including angiogenesis and inflammation [15-17]. In particular, miR-126 may play a prominent role in the control of vascular integrity and angiogenesis [18-20], as it modulates the release of angiogenic factors, such as hypoxia-inducible factor 1-alpha (HIF-1α), MMPs and vascular endothelial growth factor (VEGF), which is crucial in the development of proliferative DR [21].

Angiopoietin-2 (Ang-2) is also involved in the detachment and loss of pericytes [22-24]. Ang-2 overexpression together with VEGF increase was linked to pericyte loss and angiogenesis [25]. The Ang-2 receptor Tie-2 is mainly expressed by the endothelium [22], but evidence in the literature show that Ang-2 may also bind to integrins [26, 27].

Finally, platelet-derived growth factor B (PDGF-B), secreted by the endothelium, and its receptor PDGFR-β, which is expressed by pericytes and considered a pericyte marker, are crucial in the proliferation, migration and recruitment of pericytes to the vasculature during retinal development and in the stabilization of newly-formed vessels [23, 28].

This study was aimed at further investigating the molecular mechanisms of EV-induced vessel destabilization, by addressing miR-126 expression, the subsequent HIF-1α and VEGF modulation, Ang-2 and PDGF signalling pathways, in human retinal pericytes (HRP) after exposure to MSC-derived EV obtained in diabetic-like conditions.

Materials and Methods

Cell culture reagents were purchased from Sigma-Aldrich, unless otherwise stated. Antibodies anti-human Ang-2, Tie-2, PDGF, VEGF, HIF-1α, β-Actin were from Santa Cruz Biotechnology, anti-human PDGFR-β from Abcam, anti-human integrin α1, β1, αvβ3 by Thermo Scientific, anti-human α1-PE and β1-PE by Becton Dickinson, αvβ3-FITC-conjugated by BioLegend.

Cell cultures

HRP were immortalized and characterized in our laboratory [29]. Human bone marrow MSC were purchased from Lonza. HRP and MSC were grown in DMEM + 10%FCS. To evaluate direct miR-126 expression, HRP were grown for 8 days in physiological (5.6 mmol/L, NG), high (28.0 mmol/L, HG) or intermittent high glucose (intHG, 48h HG / 48h NG, twice).

EV isolation

MSC were cultured for 8 days in NG or HG, as above described. Hypoxic conditions were obtained by keeping cultures in a 5%CO_2 / 94%N_2 /1%O_2 gas mixture for the last 24 hrs (NG hypo, HG hypo). MSC were serum-deprived 24 hrs prior to EV isolation.

Cell debris and apoptotic bodies were removed from the supernatants by centrifugation for 30' at 3,000 and 10,000g respectively, then EV were isolated by further ultracentrifugation at 100,000g for 3 hrs at 4°C. They were either used immediately or stored at −80°C in DMEM + 5%DMSO. No differences in biological activity were observed between fresh and stored EV. EV size distribution and number were assessed using a NanoSight LM10, through the Nanoparticle Tracking Analysis 2.3 software. For all experiments an EV concentration corresponding to that found in MSC

supernatants (1×10^{11} ± 1×10^{3} EV/ml, corresponding to 1×10^{5} ± 1×10^{2} EV/cell) was used. EV internalization into HRP was checked by confocal microscopy, pre-labelling EV with the red fluorescent aliphatic chromophore PKH26 dye, as previously described [2].

RNA isolation and quantitative real-time PCR (qRT-PCR)

HRP were exposed for 6, 24 or 48h to EV obtained in the above described conditions, in serum-deprived DMEM. Total RNA was extracted from HRP and EV cultured in the above-described conditions, using mirVana RNA isolation kit (Life Technologies), which also allows for isolation of small RNAs, according to the manufacturer's instructions. After spectrophotometric quantification (Nanodrop ND-1000), 200 ng RNA were reverse-transcribed using miScript Reverse Transcription Kit (Qiagen). qRT-PCR was performed by 48-well StepOne Real Time System (Applied Biosystems) using a miScript SYBR Green PCR Kit (Qiagen). miRNAs specific primers to miR-126 and miR-296 (Life Technologies) were used. miR expression was normalized against the small nuclear RNA RNU6B.

Western blot analysis

HRP were exposed for 48h to EV obtained in the above described conditions and lysed using M-PER Mammalian Protein extraction reagent (Pierce) added with 10 µl/ml protease inhibitor cocktail kit (Pierce). Protein concentration was measured using Bradford method. 30µg proteins were loaded on pre-cast gels (4–15% Mini-PROTEAN® TGX™ Precast Gel, Biorad), separated by electrophoresis and transferred to nitrocellulose membranes. Immunoblotting was performed by incubating the membranes with specific antibodies, according to the manufacturer's instructions.
The relative signal strength was quantified by densitometric analysis (*1D Image Analysis System*, Kodak), and values normalized against β-actin.

FACS analysis

Integrin expression on HRP membranes after 48h EV stimulation was checked by FACS analysis. HRP were detached by non-enzymatic solution (Cell Dissociation Solution), incubated 20' at RT with the relevant conjugated antibody, washed and analysed at FACS (Becton Dickinson).

Statistical analysis

Results are mean ± SD of 5 independent experiments, normalized against control (NG cultures without EV). Statistical comparisons among groups were carried out by two-tailed Student's t-test for paired data or Wilcoxon's Signed Ranks test, as appropriate. Results were considered significant for $p\leq0.05$. SPSS software version 22.0 (IBM) was used for statistical analysis.

Results

EV internalization into HRP

EV were collected from the supernatant of bone marrow-derived MSC cultured in the above described conditions (NG, HG, NG hypo, HG hypo), to mimic the diabetic retinopathy milieu.

NanoSight analysis showed super-imposable concentrations and similar mean size for EV obtained in all conditions (NG: 130±23 nm, HG: 141±34 nm, NG hypo: 137±46 nm, HG hypo: 139±49 nm). EV were able to enter pericytes and locate inside the cytoplasm, as already described [2]. EV internalization into HRP did not change in the different experimental conditions.

miR-126 and miR-296 expression in EV, HRP and EV-treated HRP

EV obtained in all experimental conditions expressed miR-126, but this expression was not modulated by diabetic-like culture environment (Fig.1a). HRP also expressed miR-126, and this expression was down-regulated (-24.0%, $p<0.05$ vs NG) when they were grown in intHG conditions, while hypoxia had no direct effect (Fig. 1b). Exposure of pericytes to EV obtained in physiological conditions increased miR-126 expression (+88% after 24h exposure, $p<0.05$ vs control, +100% after 48h, $p<0.05$). However, when HRP were exposed to EV extracted from the supernatant of MSC cultured in diabetic-like conditions, their miR-126 expression was decreased, HG and hypoxic conditions playing a synergistic effect (48h: -39.4% HG-EV in comparison with NG-EG, $p<0.05$; -43.8% NGhypo-EV, $p<0.05$; -68.1% HGhypo, $p<0.05$) (Fig. 1c).

As further control, we investigated the expression of miR-296, a pro-angiomiR which stimulates VEGF receptor exposure on EC membrane [6]. HRP expressed miR-296, but this was not modulated by direct exposure of HRP to intHG/hypoxic conditions nor to EV obtained in any condition (data not shown).

VEGF and HIF-1α expression in EV-treated HRP

miR-126 is known to regulate MMPs, VEGF and HIF-1α [17]. Since we have already demonstrated MMP-2 involvement in EV-induced vessel destabilization [2], we verified VEGF and HIF-1α expression by Western blotting in HRP after EV-treatment.

We found a significant increase in VEGF content in HRP after exposure to EV obtained in diabetic-like conditions, in comparison with untreated controls (HG-EV: +98.4%, $p<0.05$; NGhypo-EV: +155.9, $p<0.05$; HGhypo-EV: +150.2, $p<0.05$) and NG-EV ($p<0.05$ in all cases) (Fig. 2a).

HIF-1α expression was also increased in HRP after exposure to EV, especially those obtained in stress conditions (NG-EV: +65.9%, $p<0.05$ vs ctrl; HG-EV: +182.8%, $p<0.05$; NGhypo-EV: +250.9, $p<0.05$; HGhypo-EV: +162.8, $p<0.05$) (Fig. 2b).

Consequently, we hypothesize that miR-126 down-regulation in pericytes following EV-treatment is responsible for the release of angiogenic/destabilizating factors, such as MMP-2, VEGF and HIF-1α.

PDGF/PDGFR-β signalling pathway

Since PDGF/PDGFR-β signalling is mostly important in pericyte recruitment and vessel stabilization, we investigated its expression in HRP after treatment with MSC-derived EV. We found a 45% decrease in PDGF expression in HRP exposed to EV in all experimental conditions in comparison with untreated pericytes ($p<0.05$), but no modulation by diabetic-like conditions (Fig. 3a). No differences were found in PDGFR-β expression (Fig. 3b).

Ang-2 signalling pathway

Ang-2 signalling is also involved in the detachment and loss of pericytes [22]. We therefore investigated if EV may modulate the expression of Ang-2 and its receptors (Tie-2 and integrins) in pericytes. In our experimental setting, HRP expressed Ang-2, but our results demonstrated no EV modulation. Both Western blotting and FACS analysis showed that HRP do not express Tie-2 receptor. As regards integrins, we found that HRP express the β1 subunit in all experimental conditions, while α1 and αv/β3 were undetectable by both Western blot and FACS. We can therefore exclude an involvement of Ang-2 and PDGF signalling pathways in EV-induced vessel destabilization.

Discussion

We have recently demonstrated that MSC-derived EV are able to enter pericytes, causing detachment from their substrate and migration, possibly leading to loss of control on EC proliferation and, consequently, angiogenesis and increased blood-barrier permeability. These effects are mediated by MMP-2, expressed by both EV and EV-stimulated pericytes, and enhanced if EV had been obtained in diabetic-like conditions [2].

In the present work, we show for the first time a role of miR-126 in EV-induced vessel destabilization. MSC-derived EV obtained in hyperglycaemic/hypoxic conditions down-regulated miR-126 expression in pericytes leading to increased expression of angiogenic molecules, such as VEGF and HIF-1α.

MicroRNAs are short and highly-preserved RNA sequences, not encoding for any protein but acting as powerful gene modulators and playing important roles in many biological processes, such as cell proliferation, differentiation and apoptosis [15]. In particular, they are involved in angiogenesis and inflammation [17, 19] and a role for them in diabetic complications has been postulated [17, 30, 31]. miR-126 is primarily expressed in EC and hematopoietic progenitor cells and is commonly considered a pro-angiomiR, as it has a prominent role in the control of vascular integrity and angiogenesis [18-20]. In this work, we show for the first time at our knowledge that miR-126 is expressed also in pericytes. Consistently with the above described findings, our data suggest that treatment of HRP with EV obtained in physiological conditions enhances their miR-126 expression. However, type 2 diabetic subjects show a loss of endothelial miR-126 [32], while a down-regulation of miR-126 was observed in experimental models of DR [33, 34], as well as in diabetic retina extracts and in chorioretinal EC in hypoxic conditions [34]. This correlates with an increase in angiogenic factors such as HIF-1α, MMPs and especially VEGF, which is known to be crucial in the development of proliferative DR and diabetic macular oedema [17, 21, 34]. Moreover, restoration of miR-126 levels was found to down-regulate the expression of VEGF, HIF-1α and IGF, leading to reduced retinal neovascularization in ischemic conditions [33]. Our results, showing down-regulation of miR-126 in HRP exposed to EV obtained in hyperglycaemic and hypoxic conditions, together with the subsequent increase in VEGF, HIF-1α and, as previously observed, MMP-2 [2], are consistent with those reports. In addition, we show a direct, non EV-mediated, decrease of miR-126 in HRP cultured in a hyperglycaemic-like milieu. We can therefore hypothesize that down-regulation of miR-126 in a diabetic-like microvascular milieu may not only indirectly stimulate EC proliferation, through the increased release of angiogenic factors, but also contribute to early pericyte loss and vessel destabilization. This new findings may possibly open new therapeutic scenario for the prevention of DR, through the treatment with miR-126 antagonists.

PDGF signalling pathway play a key role in the mechanisms underlying vascular changes in DR. PDGF-B, secreted mostly by endothelium, and its receptor PDGFR-β, expressed by pericytes [28] are in fact crucial in the proliferation, migration and recruitment of pericytes during retinal development, and pericyte coverage is essential for the stabilization of immature endothelial tubes [35, 36]. In diabetic mouse retinas, PDGF-B mRNA is decreased in comparison with non-diabetic mice, suggesting a role for PDGF-B in hyperglycaemia-induced pericyte loss [28]. Therefore, we investigated the possible involvement of this signalling pathway in the EV-induce vessel destabilization. Our findings, however, show no significant difference in PDGFR-β expression on pericyte surface as a consequence of EV exposure, thus ruling out this pathway from the signalling mechanisms through which MSC-derived EV cause vessel destabilization.

Moreover, our results permit to rule out an involvement of Ang-2 signalling in EV-induced pericyte destabilization. Ang-2 is involved in pericyte loss and subsequent angiogenesis, and a synergistic action with VEGF has been postulated [25, 37]. Conversely, Ang-2 overexpression in the absence of VEGF may lead to vasoregression [25]. Tie-2 receptor is expressed mainly on EC membranes [22] and our data confirm that it is not expressed by pericytes. However, it has been shown that Ang-2 may act via Tie-2 independent pathways, in particular through integrins [26, 27]. We therefore checked for integrin expression on pericytes in our experimental settings but could only demonstrate the presence of the integrin β1, the expression of which is not modulated by exposure to EV. Consequently, in our experimental setting, Ang-2 itself was not influenced by EV-treatment of HRP.

In conclusion, we hypothesize that MSC-derived EV obtained in diabetic-like conditions are able to determine pericyte detachment, and, possibly, vessel destabilization, through down-regulation of miR-126. This, in turn, may lead to increased expression of angiogenic factors, such as VEGF, HIF-1α and MMP-2, responsible for pericyte detachment, loss of control on endothelial proliferation and, finally, angiogenesis and retinal neovascularization.

The role of miRNAs in the development of DR is still little understood, and probably other miRNAs involved are yet to be discovered, but in the near future a treatment with miRNA agonists or antagonists to prevent or slow the progression of the disease could be hypothesized. In particular, a strategy to increase miR-126 expression in diabetic retinal microvessels could be outlined. Restoration of miR-126 levels could resulting in the inhibition of damaging factors, such as VEGF and HIF-1α.

Further research is also needed to deeper investigate the location and role of MSC-derived EV in the pathogenesis of DR.

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Conflict of interest

Aurora Mazzeo, Elena Beltramo, Alessandra Iavello, Andrea Carpanetto and Massimo Porta declare that they have no conflicts of interest.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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Figures

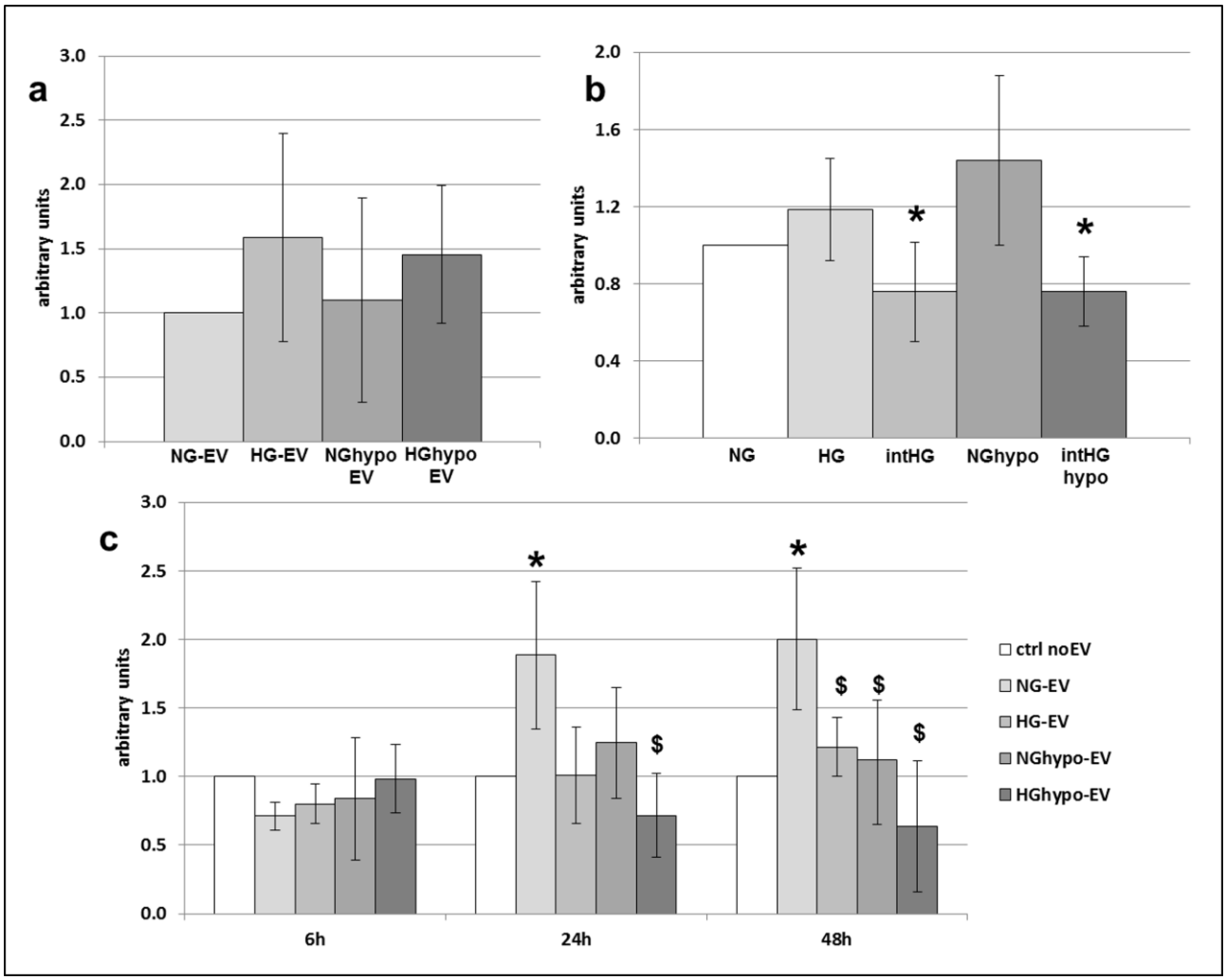

Fig.1 miR-126 expression (qRT-PCR) in: **a**) MSC-derived EV, **b**) HRP cultured directly in diabetic-like conditions (* $p<0.05$ vs physiological conditions, NG), **c**) HRP exposed for 6, 24 and 48h to MSC-derived EV obtained in physiological or diabetic-like conditions, as compared to untreated control (white bar, HRP in physiological conditions without EV-treatment, * = $p<0.05$ vs ctrl noEV, $ = $p<0.05$ vs NG-EV)

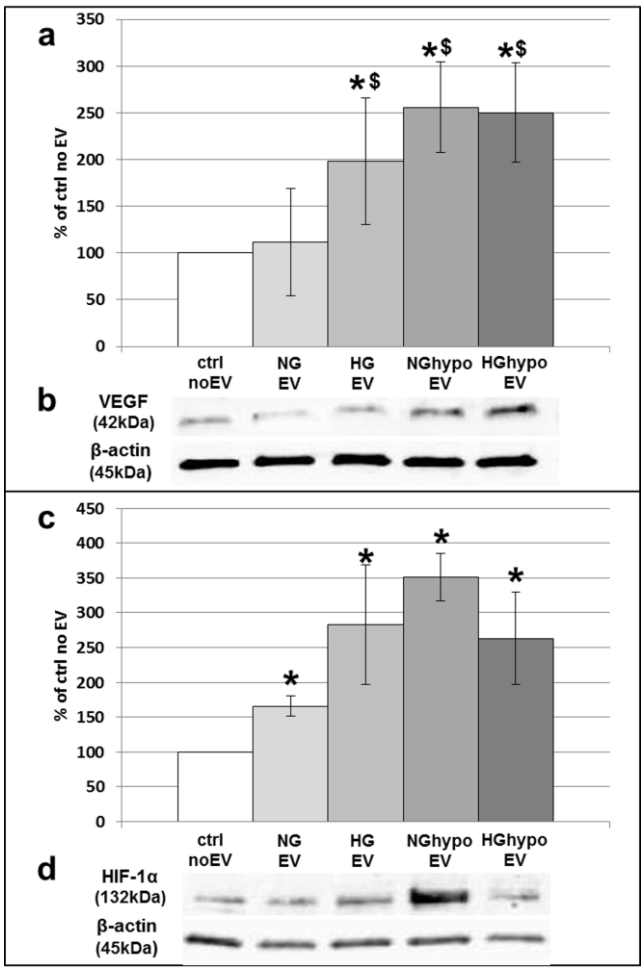

Fig. 2 VEGF (**a-b**) and **HIF-1α** (**c-d**) **expression** in HRP following EV stimulation. **a-c**) Quantitative analysis of band densities after Western blotting, percentages of untreated control. *p<0.05 vs ctrl noEV, $ p<0.05 vs NG-EV, **b-d**) images of one representative gel

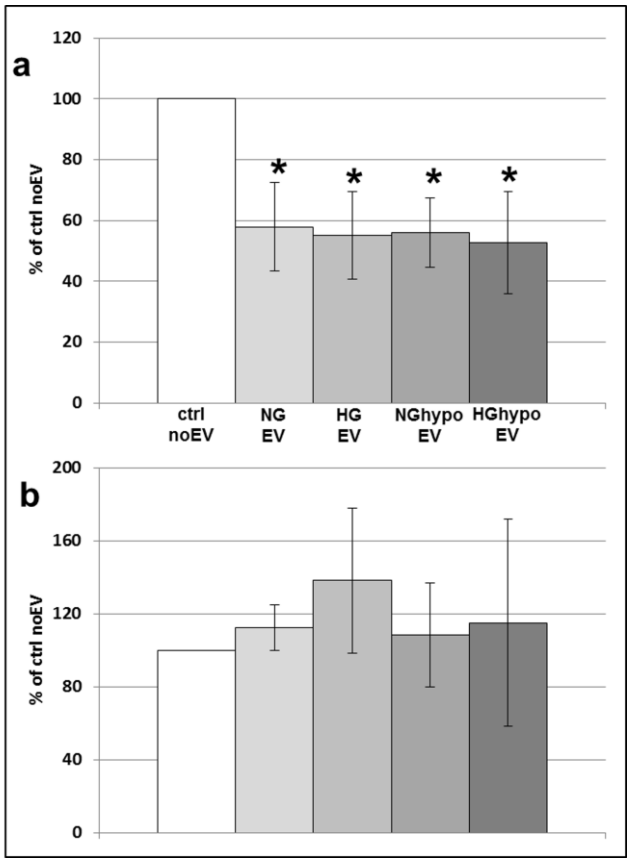

Fig. 3 PDGF (**a**) and **PDGFR-β** (**b**) expression in HRP following EV stimulation. Quantitative analysis of band densities after Western blotting, percentages of untreated control. *p<0.05 vs ctrl noEV