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Platelet-derived growth factor BB stimulates vasculogenesis of embryonic stem cell-derived endothelial cells by calcium-mediated generation of reactive oxygen species

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Angiogenesis;
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Reactive oxygen species;
Vasculogenesis

Aims Platelet-derived growth factor BB (PDGF-BB) has been assigned a critical role in vascular growth and recruitment of perivascular mural cells. The purpose of the present study is to investigate the signalling events underlying the stimulation of vasculogenesis of mouse embryonic stem (ES) cells by PDGF-BB.

Methods and results PDGF-BB increased vascular sprouting and branching of capillary-like structures in embryoid bodies as evaluated by computer-assisted analysis of CD31-positive cell structures. It also activated extracellular-regulated kinase 1,2 (ERK1,2) and c-Jun N-terminal kinase but not p38 mitogen-activated protein kinase or PI 3-kinase. Microfluorometric analysis of fluo-4 fluorescence revealed that treatment with PDGF-BB raised intracellular Ca^{2+} levels in differentiating ES cells expressing the PDGF receptor β, an effect that was abolished in the presence of the intracellular Ca^{2+} chelator BAPTA. Furthermore, PDGF-BB raised reactive oxygen species (ROS) levels in embryoid bodies as evaluated using the redox-sensitive dye H_2DCF-DA. ROS generation was blunted in the presence of the NADPH oxidase inhibitors diphenylen iodonium (DPI) and apocynin as well as in the presence of BAPTA, suggesting that ROS generation is regulated by intracellular Ca^{2+} transients. The stimulation of vasculogenesis of ES cells upon treatment with PDGF-BB was significantly inhibited by the ERK1,2 inhibitor U0126, the NADPH oxidase inhibitors DPI, apocynin, 4-(2-aminoethyl)benzenesulfonylfluoride and VAS2870, the free radical scavengers vitamin E, and N-(2-mercaptopropionyl)glycin as well as by BAPTA.

Conclusion Our data demonstrate that the pro-vasculogenic effects of PDGF-BB are mediated by Ca^{2+}-induced ROS generation, resulting in the activation of an ERK1,2-mediated signal transduction cascade.

1. Introduction

The peptide growth factor platelet-derived growth factor (PDGF) consists of different combinations of two polypeptide chains, A and B, which form homodimeric (PDGF-AA and PDGF-BB) as well as heterodimeric (PDGF-AB) isoforms binding to two structurally related protein tyrosine kinase receptors, namely α- and β-receptors. Recently, two new PDGF isoforms, namely PDGF-CC[1] and PDGF-DD,[2] were identified. Whereas PDGF-CC can bind to PDGFR-αα and PDGFR-αβ receptors, PDGF-DD has been reported to activate PDGFR-αβ and PDGFR-ββ. Binding to their cognate receptors results in the activation of signalling cascades that initiate proliferation, migration, and differentiation of a variety of cell types including fibroblasts and smooth muscle cells.[3] PDGF-BB has been previously assigned a critical role in the maintenance of vascular stability through attraction of mural cells expressing the PDGF receptor-β (PDGFR-β).[4] Besides its role in the recruitment of pericytes to the endothelial cell layers, pro-angiogenic effects of PDGF-BB have been reported to occur in physiological[5] as well as patho-physiological conditions, e.g. during myocardial infarction[6] and during tumour vascularization.[7] PDGF-BB and PDGFR-β are mainly expressed in the developing vasculature, where PDGF-BB is produced by endothelial cells and PDGFR-β is expressed by mural cells, including

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pericytes.[8] During angiogenic processes, members of the PDGF family act in concert with other pro-angiogenic factors, e.g. FGF-2, to induce angiogenic synergy and vessel stability, suggesting that combinatorial therapy with angiogenic and arteriogenic factors may be utilized in therapeutic angiogenesis.[9] Recently, it has been shown that stimulation of PDGFR-β induced sprouting vasculogenesis in differentiating embryonic stem (ES) cells,[10] which form capillary-like structures within the three-dimensional tissue of embryoid bodies and express the endothelial cell markers Flk-1 (VEGF-R2), flt-1 (VEGF-R1), CD31, CD34, Tie-1, Tie-2, and VE-cadherin.[11–13] Haemangioblastic precursor cells expressed PDGFR-β, and PDGF-BB treatment decreased the expression of markers for primitive and definitive haematopoiesis, thus suggesting that PDGF-BB treatment of haemangioblastic precursors stimulated sprouting vasculogenesis at cost of haematopoietic commitment. PDGFR-β expression on endothelial cells was lost during later stages of endothelial cell differentiation, thus suggesting that PDGF-BB accelerates the differentiation of endothelial cells.[10] The signalling pathways by which PDGF-BB stimulates vasculogenesis of ES cells are not yet known. Previous studies performed on smooth muscle cells have demonstrated that PDGF-BB treatment induced an elevation of intracellular reactive oxygen species (ROS), which initiated cell proliferation and migration.[14] Further studies have demonstrated that PDGF-BB elicited a transient Ca^{2+} response in smooth muscle cells, which is likewise involved in cell migration.[15] The present study was undertaken to unravel the signalling cascades underlying the pro-vasculogenic effects of PDGF-BB in differentiating mouse ES cells. It is demonstrated that PDGF-BB effects on vasculogenesis are dependent on both Ca^{2+} and ROS signals and involve activation of the extracellular-regulated kinase 1,2 (ERK 1,2) mitogen-activated protein kinase (MAPK) pathway.

2. Methods

2.1 Spinner-culture technique for cultivation of embryoid bodies

ES cells (line CCE) were grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts as previously described.[16] At day 0 of differentiation, adherent cells were enzymatically dissociated and seeded at a density of 3×10^6 cells mL^{-1} in 250 mL siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 125 mL LIF-free Iscoves medium. Following 24 h, 125 mL medium was added to give a final volume of 250 mL. The spinner flask medium was stirred at 20 r.p.m. using a stirrer system (Integra Biosciences). Within spinner flasks, ES cells formed three-dimensional spherical cell aggregates (0.5–1 mm in diameter), named embryoid bodies, in which ES cells differentiated into cells of all three germ layers.

2.2 Treatment of embryoid bodies with PDGF-BB, PDGF-AA, VEGF-A, and other agents

To investigate the effects of PDGF-BB, PDGF-AA, and VEGF-A on vasculogenesis of ES cells, 4-day-old embryoid bodies were transferred to bacteriological cell culture dishes (diameter 60 mm) filled with 5 mL differentiation medium comprising serum-free Iscoves cell culture medium supplemented with 15% knock-out serum-replacement medium (Invitrogen, Karlsruhe, Germany). They remained either untreated (controls) or were treated with different concentrations of PDGF-BB (R&D Systems, Wiesbaden, Germany), PDGF-AA (Milteny Biotech, Bergisch Gladbach, Germany), and VEGF-A (Milteny Biotech) from day 4 to day 8 of cell culture. Radical scavengers, i.e. vitamin E (Trolox) (Calbiochem, Bad Soden, Germany) and *N*-(2-mercaptopropionyl)glycin (NMPG) (Sigma), NADPH oxidase inhibitors, i.e. diphenylen iodonium (DPI) (Sigma), apocynin (Calbiochem), 4-(2-aminoethyl)benzenesulfonyl-fluoride (AEBSF) (Sigma), and 3-benzyl-7-(benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine (VAS2870) (Vasopharm, Würzburg, Germany), and the ERK1,2 inhibitor U0126 (Calbiochem) were pre-incubated for 2 h prior to the addition of PDGF-BB to the incubation medium.

2.3 Recording of intracellular Ca^{2+} concentrations

Single cell preparations were prepared by enzymatic dissociation of either undifferentiated ES cells or embryoid bodies at different stages of differentiation as indicated. Briefly, tissues were digested for 30 min at 37°C in phosphate-buffered saline (PBS) containing 2 mg/mL Collagenase B (Boehringer, Ingelheim, Germany). After attachment to cover slips, cells were loaded in serum-free medium with 10 μM Fluo-4/AM (Molecular Probes, Eugene, OR, USA) for 30 min and transferred to the incubation chamber of a confocal laser scanning microscope (Leica SP2, AOBS, Leica, Bensheim, Germany). Fluorescence excitation was performed at 488 nm, and emission was recorded at 500–550 nm. Changes in fluorescence values were presented as fluorescence F in relation to the baseline fluorescence F_0.

2.4 Measurement of reactive oxygen species generation

Intracellular ROS levels were measured using the fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes). For the experiments, embryoid bodies were incubated in serum-free medium and treated for 2 h with 10 ng/mL PDGF-BB. Subsequently 20 μM H_2DCF-DA dissolved in dimethyl sulfoxide was added. After 30 min, intracellular DCF fluorescence was evaluated in 3600 μm^2 regions of interest using an overlay mask. To monitor ROS generation directly after PDGF-BB administration, embryoid bodies were loaded for 30 min with 20 μM H_2DCF-DA. DCF generation was evaluated in 10 s time intervals prior and following PDGF-BB addition to the incubation medium. For fluorescence excitation, the 488 nm band of the argon ion laser of the confocal setup was used. Emission was recorded using an emission band of 515–550 nm.

2.5 Immunohistochemistry

As primary antibodies, the rat monoclonal anti-CD31 (Millipore, Schwalbach, Germany) (dilution 1: 100), the rabbit polyclonal anti-p-ERK1,2, anti-p-JNK, anti-p-p38, and anti-p-PI3K (p85/p55) antibodies, directed against the phosphorylated form of the proteins (New England Biolabs, Frankfurt, Germany) (dilution 1:100) were used. For CD31 staining, the respective tissues were fixed in ice-cold methanol for 1 h at −20°C and washed with PBS containing 0.01% Triton X-100 (PBST) (Sigma). For ERK1,2, c-Jun N-terminal kinase (JNK), and p38 the tissues were fixed for 20 min at room temperature in 4% formaldehyde in PBS, subsequently washed once in PBS and fixed for additional 20 min in methanol (−20°C). Blocking against unspecific binding was performed for 60 min with 10% FCS dissolved in 0.01% PBST. For CD 31 staining, tissues were incubated for 2 h at room temperature with primary antibody (dilution 1:200) dissolved in 0.01% PBST supplemented with 10% FCS. Staining for ERK1,2, JNK, and p38 (dilution 1:100) was performed at 4°C overnight. The tissues were thereafter washed three times with PBST and re-incubated with either a Cy5-conjugated goat anti-rat IgG (H+L) (CD31) or a Cy5-conjugated goat anti-rabbit IgG (ERK1,2, JNK, p38) (all from Dianova, Hamburg, Germany) at a 1:200 dilution in 0.01% PBST supplemented with 10% FCS. Fluorescence recordings were performed by confocal laser scanning microscopy using a 5 mW helium/neon laser, single

excitation 633 nm (excitation of Cy5). Emission was recorded at >665 nm. The pinhole settings of the confocal setup were adjusted to give a full-width at half-maximum of 10 μm.

2.6 Quantification of vasculogenesis in embryoid bodies

For the quantification of capillary areas within embryoid bodies, an optical sectioning routine based on confocal laser scanning microscopy was used. Images (512 × 512 pixels) were acquired from CD31-stained embryoid bodies using the extended depth of focus algorithm of the confocal setup. In brief, five full-frame images separated by a distance of 20 μm in the *z*-direction were recorded, which included the information of the capillary area and spatial organization of a 100 μm thick tissue slice. From the acquired images, an overlay image giving a three-dimensional projection of the vascular structures in the scanned tissue slice was generated. By use of the image analysis facilities of the confocal setup the branching points of vascular structures within the three-dimensional projection of vascular structures were identified and counted in relation to the size of the respective embryoid body.

2.7 Real-time RT–PCR

Total RNA from CCE embryoid bodies was prepared using the Trizol (Invitrogen) method, followed by genomic DNA digestion using DNase I (Invitrogen). Total RNA concentration was determined by the OD_{260nm} method. cDNA synthesis was performed using 2 μg RNA with MMLV RT (Invitrogen). Primer concentration for qPCR was 6 pmol, and primer (Invitrogen) sequences are given in *Table 1*.

Amplifications were performed in an ICycler Optical Module (Biorad, Munich, Germany) using iQ™ SYBR Green Supermix (Biorad). Following programmes were used:

Cycle 1 (1×): Step 1: 95°C for 3 min;
Cycle 2 (45×); Step 1: 95°C for 30 s;
Step 2: 61.5°C for 30 s (annealing temperature);
Step 3: 72°C for 30 s;
Cycle 3: Step 1: 50°C for 10 min.

Relative expression values were obtained by normalizing C_T values of the tested genes in comparison with C_T values of the housekeeping genes using the $\Delta\Delta C_T$ method.

2.8 Immunoblotting

For immunoblotting, 4-day-old PDGF-BB treated embryoid bodies were washed twice with ice-cold PBS before being lysed in RIPA buffer [50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate] containing 1% phosphatase inhibitor cocktail 1 (Sigma), and 0.2% protease inhibitor cocktail (Biovision, Hannover, Germany). Samples (40 μg protein) were subjected to gel electrophoresis on NUPAGE® 4–12% Bis–Tris Mini gels (Invitrogen). Gels were transferred to polyvinylidene difluoride membranes by iBlot™ system (Invitrogen). Antibodies used were anti-p44/42 MAP kinase, anti-phospho-p44/42 MAP kinase, anti-p38 MAP kinase, anti-phospho-p38 MAPK, anti-PI3K p85, anti-phospho-PI3K p85/p55, anti-SAPK/JNK, anti-phospho-SAPK/JNK (Cell Signaling), and anti-vinculin (Sigma). Protein bands were detected by horseradish peroxidase-labelled anti-rabbit (Dianova) or anti-mouse (Cell Signaling) antibodies using of the ECL detection system (Thermo Fischer Scientific, Rockford, IL, USA).

Table 1 Primer sequences for vascular- and smooth muscle-associated genes.

Pol 2A	Forward 5′-GAC AAA ACT GGC TCC TCT GC-3′
	Reverse 5′-GCT TGC CCT CTA CAT TCT GC-3′
CD31	Forward 5′-GTC ATG GCC ATG GTC GAG TA-3′
	Reverse 5′-CTC CTC GGC GAT CTT GCT GAA-3′
VE-cadherin	Forward 5′-GTC AGC TAT AGG GAC CTC TGT-3′
	Reverse 5′-TCA TTT CCT TTC ACG ATT TGG-3′
α-SM-actin	Forward 5′-CTG ACA GAG GCA CCA CTG AA-3′
	Reverse 5′-CAT CTC CAG AGT CCA GCA CA-3′
Caldesmon	Forward 5′-GCC TCA GGA ACA CCC AAT AA-3′
	Reverse 5′-GTT CCG CTT GCC AGA TAC AT-3′
h-Calponin	Forward 5′-TAC ACG GCG TCA CCT CTA TG-3′
	Reverse 5′-AGT GTG TCG CAG TGT TCC AT-3′
SM22	Forward 5′-GTG GCT GAA GAA TGG TGT GA-3′
	Reverse 5′-ATT GAG CCA CCT GTT CCA TC-3′

2.9 Flow cytometry

Embryoid bodies (day 4) were separated into single-cell suspension by incubation for 30 min in collagenase B solution (2.0 mg/mL) (Boehringer Mannheim, Germany). A total of 17×10^6 cells were either stained for PDGFR-β (2 μg) (Natutec, Frankfurt, Germany) or for the appropriate rat IgG isotype control (2 μg) (BD Biosciences, Heidelberg, Germany). After washing, cells were incubated with goat-anti-rat IgG PE-Cy5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For CD31 cells were stained with the direct conjugated antibody CD31-PE (3 μg) (BD Biosciences) or the appropriate PE-rat IgG (Biozol Diagnostica, Munich, Germany) isotype control. After washing three times with PBS, cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

2.10 Statistical analysis

Data are given as mean ± SD, with *n* denoting the number of experiments unless otherwise indicated. One-way ANOVA for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

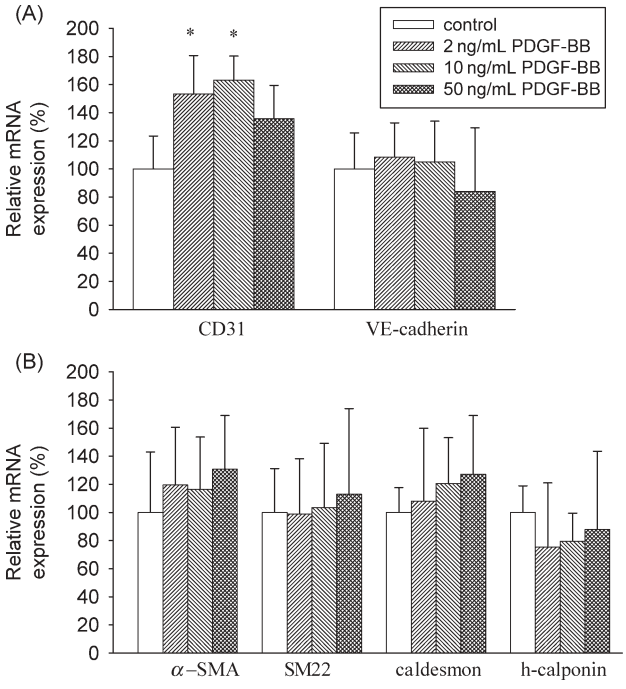

Figure 1 Expression of endothelial (*A*) and smooth muscle mRNA (*B*) upon treatment with different concentrations of PDGF-BB as evaluated by real-time RT–PCR. Embryoid bodies were treated from day 4 to day 8 of cell culture with PDGF-BB. mRNA preparation was performed on day 8. The data are presented as relative mRNA expression normalized to the expression of the house-keeping gene Pol2A. *$P < 0.05$, significantly different from the untreated control.

3. Results

3.1 Analysis of the effects of PDGF-BB on the expression of endothelial and smooth muscle cell markers in embryoid bodies grown from ES cells

To validate the pro-vasculogenic effects of PDGF-BB in CCE, ES cells embryoid bodies were treated from day 4 to day 8 of cell cultures with different concentrations of PDGF-BB ranging from 2 to 50 ng/mL and the mRNA expression of the endothelial cell markers CD31 and VE-cadherin (*Figure 1A*, $n = 4$) as well as the smooth muscle markers α-SMA, SM22, caldesmon, and h-calponin (*Figure 1B*, $n = 4$) was investigated. It was demonstrated that PDGF-BB treatment significantly increased the expression of CD31 mRNA, whereas VE-cadherin expression and the expression of smooth muscle cell-associated genes was not significantly changed. Image analysis of CD31-positive cell areas revealed that the degree of vascularization of embryoid bodies significantly increased with the dose of administered PDGF-BB (*Figure 2A*; $n = 3$). Maximum vascularization was achieved with 10 ng/mL, whereas a decline in vascularization was apparent with 50 ng/mL PDGF-BB. This value was, however, still increased by $\sim$100% when compared with the untreated control. Comparably, treatment of embryoid bodies from day 4 to day 8 of cell culture with different concentrations of VEGF, ranging from 5 to 50 ng/mL, significantly increased vascularization (*Figure 2B*, $n = 3$). Incubation of embryoid bodies with PDGF-AA in concentrations ranging from 2 to 50 ng/mL failed to stimulate the degree of vascularization of ES cells (data not shown).

3.2 Generation of reactive oxygen species in embryoid bodies following treatment with PDGF-BB, and impact of reactive oxygen species for PDGF-BB-induced vasculogenesis

Differentiating embryoid bodies have previously been shown by us to express different NOX isoforms capable of generating ROS that are involved in cardiovascular differentiation processes.[17] To investigate ROS generation in differentiating embryoid bodies and to assess sources of ROS generation, 4-day-old embryoid bodies prior to the onset of vasculogenesis were treated with 10 ng/mL PDGF-BB and ROS generation was monitored either immediately after treatment (*Figure 3A*, $n = 3$) or 2 h following stimulation using the redox-sensitive fluorescence dye H_2DCF-DA (*Figure 3B*, $n = 3$). Treatment with PDGF-BB significantly increased ROS generation in embryoid bodies within few minutes (*Figure 3A*) which was

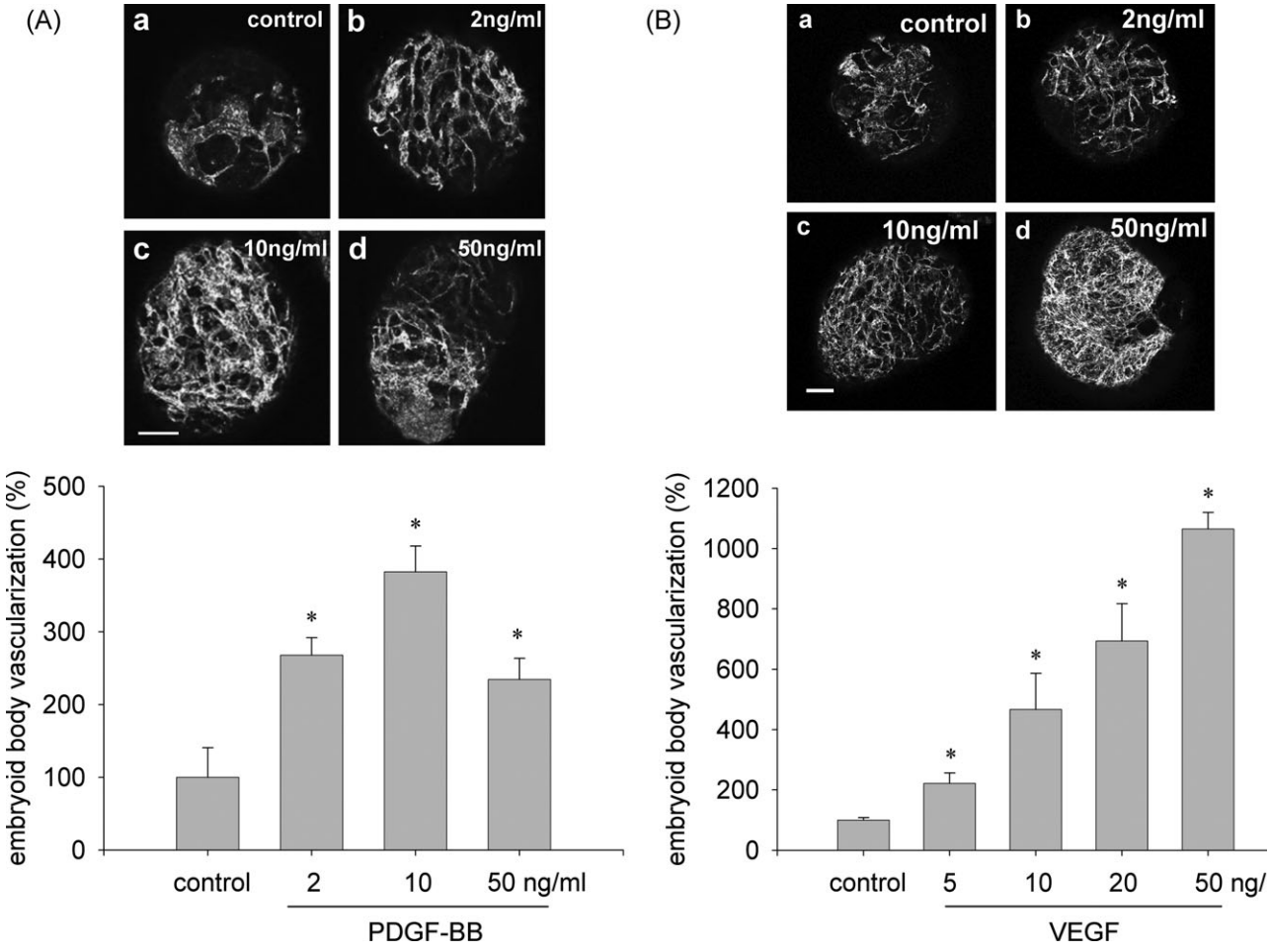

Figure 2 Effects of different concentrations of PDGF-BB (*A*) and VEGF-A (*B*) on vascularization of embryoid bodies. The upper panel shows representative embryoid bodies immunostained for CD31 expression which remained either untreated (controls) (a) or were treated from day 4 to day 8 of cell culture with either 2 ng/mL (b), 10 ng/mL (c) and 50 ng/mL (d) PDGF-BB (*A*) or 2 ng/mL (b), 10 ng/mL (c), and 50 ng/mL VEGF-A (*B*). Control samples were grown under the same cell culture conditions, but remained untreated. The bar represents 200 μm. The lower panel shows the quantitative evaluation of CD31 positive vascular structures after treatment with either PDGF-BB or VEGF-A. *$P < 0.05$, significantly different from the untreated control.

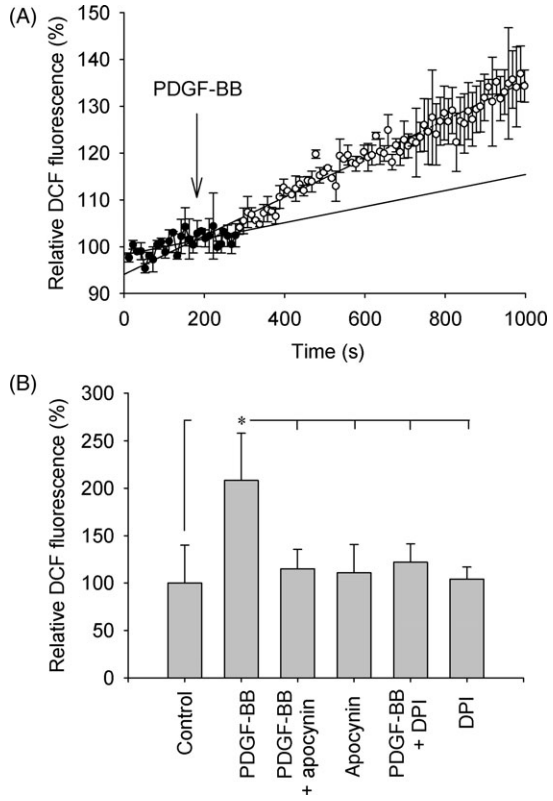

Figure 3 Generation of ROS upon treatment of embryoid bodies with PDGF-BB (10 ng/mL). (*A*) 4-day-old embryoid bodies were loaded with the redox-sensitive indicator H_2DCF-DA for 30 min and DCF fluorescence indicative for ROS generation was monitored every 10 s. At the time indicated by the vertical arrow, PDGF-BB was added to the incubation medium. The curves recorded either in the absence of PDGF-BB (black circles) or in the presence of PDGF-BB (open circles) were fitted by linear regression. Note, that the slope of the linear regression curve is increased in the presence of PDGF, indicating stimulation of ROS generation. (*B*) Effects of the NADPH oxidase inhibitors apocynin (10 μM) or DPI (1 μM), after a 2 h incubation period with PDGF-BB and a 30 min incubation period with H_2DCF-DA. DCF fluorescence was monitored in 3600 μm^2 areas within the tissue. The increase in ROS generation upon treatment with PDGF-BB was totally abolished in the presence of NADPH oxidase inhibitors. *$P < 0.05$, significantly different as indicated.

totally abolished in the presence of the NADPH oxidase inhibitors DPI (1 μM) and apocynin (10 μM) (see *Figure 3B*), thus suggesting that ROS were generated through NADPH oxidase activity. The impact of NADPH oxidase inhibition on PDGF-BB-induced vasculogenesis of ES cells was investigated by assessing the degree of vascularization in either the absence or the presence of DPI (0.5 μM), apocynin (10 μM), AEBSF (200 μM), and VAS2870 (50 μM). All applied inhibitors of NADPH oxidase abolished the stimulation of vasculogenesis achieved with PDGF-BB (*Figure 4A*–C, $n = 3$). Furthermore, pre-incubation with the free radical scavengers vitamin E (10 μM) and NMPG (10 μM) significantly inhibited PDGF-BB-mediated vasculogenesis (*Figure 4D*, $n = 3$), which clearly demonstrates that PDGF-BB exerts its pro-vasculogenic effects via NADPH oxidase-derived ROS generation.

3.3 PDGF-BB-induced Ca^{2+} signalling and its relation to vasculogenesis and reactive oxygen species generation

Previous studies have shown that PDGF-BB raises intracellular Ca^{2+} in several preparations.[18] To investigate whether PDGF-BB elicited Ca^{2+} responses, undifferentiated ES cells as well as embryoid bodies of increasing differentiation states were dissociated and intracellular Ca^{2+} was recorded in single cells. In undifferentiated ES cells, Ca^{2+} responses upon PDGF-BB treatment were almost absent. Until day 3 of differentiation, $<5\%$ of the total cells responded towards PDGF-BB treatment (*Figure 5A*), which could be abolished in the presence of the intracellular Ca^{2+} chelator BAPTA/AM (10 μM) (*Figure 5B*, $n = 3$). On day 4 of differentiation, $\sim$20% of the cells responded towards PDGF-BB treatment, which declined during subsequent days (days 5–8) to values between 10 and 15% (*Figure 5C*, $n = 3$). To correlate the cells displaying Ca^{2+} responses towards PDGF-BB treatment to the percentage of CD31-positive cells and cells positive for PDGFR-β, FACS analysis was performed with cells from 4-day-old embryoid bodies (*Figure 5D*, $n = 3$). It was shown that $28.3 \pm 9.1\%$ of the total cells were positive for CD31, $37.9 \pm 10.2\%$ were positive for PDGFR-β, and $20.7 \pm 8.5\%$ of the cells were positive for both CD31 and PDFGR-β. From the cell population positive for CD31, 73% were double-positive for PDFGR-β. These data demonstrated that the percentage of PDFGR-β- and CD31-positive cells was in good correlation with the percentage of cells showing Ca^{2+} responses towards PDGF-BB stimulation.

To study the impact of intracellular Ca^{2+} responses on PDGF-BB-induced vasculogenesis, 4-day-old embryoid bodies were treated until day 8 of differentiation with PDGF-BB in the absence and presence of BAPTA/AM as well as with BAPTA/AM alone. It was shown that BAPTA/AM treatment significantly inhibited the effect of PDGF-BB on vascularization (*Figure 6A*, $n = 3$), suggesting the involvement of intracellular Ca^{2+} signals in this effect. Furthermore, incubation of 4-day-old embryoid bodies with PDGF-BB in the presence of BAPTA/AM totally abolished the increase in ROS generation observed with PDGF-BB alone (*Figure 6B*), which indicates that PDGF-BB-induced Ca^{2+} signals are regulating NADPH oxidase activity.

3.4 Effects of PDGF-BB on mitogen-activated protein kinase pathway activation

When 4-day-old embryoid bodies were treated with PDGF-BB (10 ng/mL) an increase in ERK1,2 phosphorylation was observed, which was significantly different when compared with the untreated control after 5 min and reached maximum values after 10 min. Until 240 min after stimulation with PDGF-BB, ERK1,2 phosphorylation remained at an elevated plateau (*Figure 7A*, $n = 4$). In contrast, immunohistochemistry revealed no significant activation of p38 MAPK, JNK, and PI 3-kinase (PI3K) upon treatment with PDGF-BB (data not shown). These data were corroborated by immunoblot analyses (*Figure 7B*, $n = 3$), which demonstrated robust activation of ERK1,2 but no activation for p38 and PI3K, whereas a weak (non-significant) activation of JNK was observed. The absolute protein levels of MAPKs and PI3-kinase remained unchanged upon treatment with PDGF-BB (see *Figure 7B*). To investigate the impact of the ERK1,2 MAPK signalling cascade on PDGF-BB-mediated vasculogenesis, the degree of vascularization was assessed either in the presence or the absence of the specific ERK1,2 inhibitor U0126 (10 μM). The ERK1,2 inhibitor U0126 totally abolished the pro-vasculogenic effect of PDGF-BB (*Figure 7C*, $n = 3$), whereas vasculogenesis in the

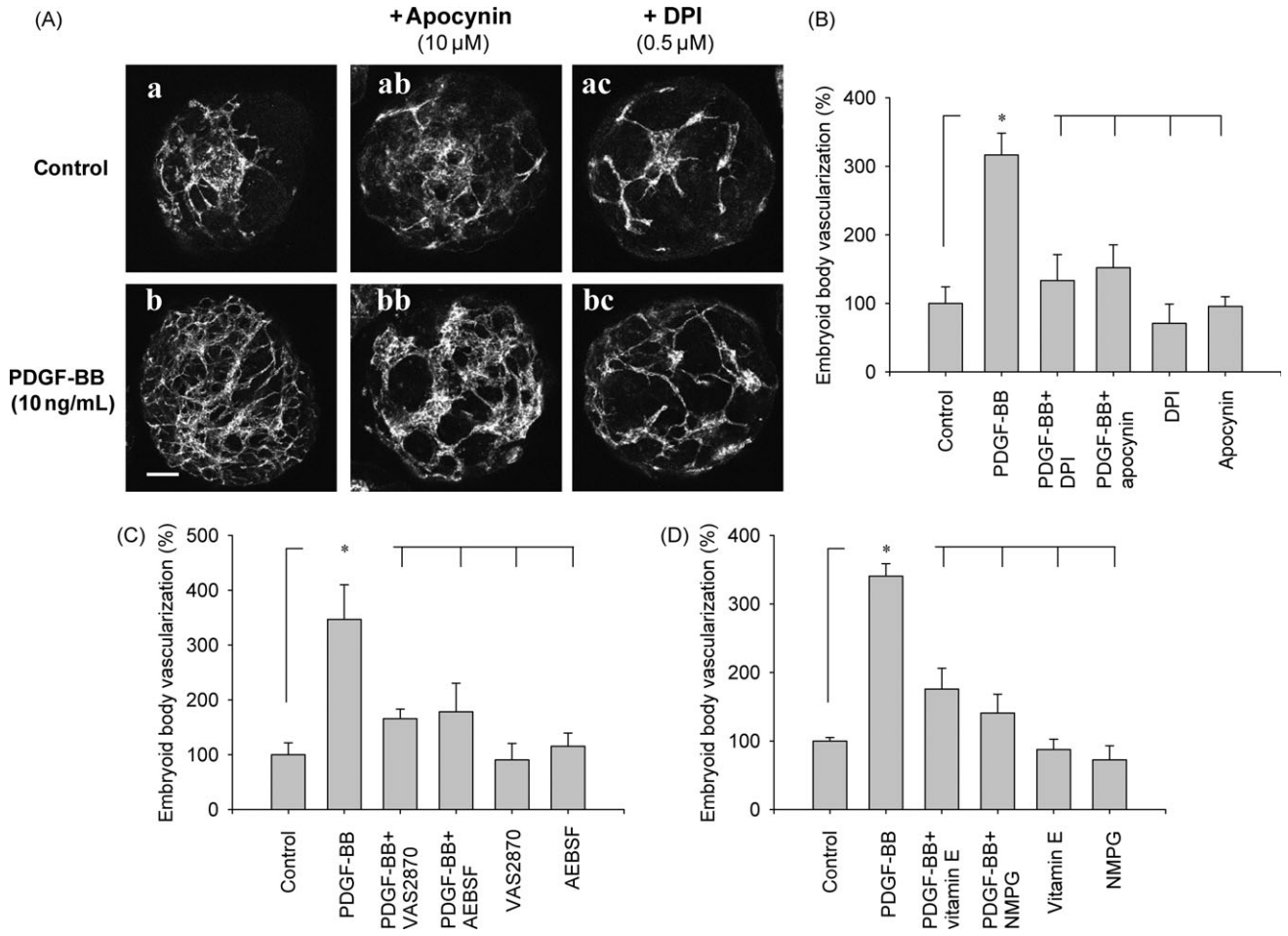

Figure 4 Inhibition of the pro-vasculogenic effects of PDGF-BB upon treatment with NADPH oxidase inhibitors and free radical scavengers. (*A*) Embryoid bodies immunostained for CD31: (a) control, (ab) treated with apocynin, (ac) treated with DPI, (b) PDGF-BB-treated, (bb) treated with PDGF-BB in the presence of apocynin, and (bc) treated with PDGF-BB in the presence of DPI. The bar represents 200 μm. (*B* and *C*) Quantification of CD31-positive vascular structures under conditions where embryoid bodies remained either untreated (control) or were treated with either apocynin (10 μM) or DPI (1 μM) (*B*) or with either VAS2870 (50 μM) or AEBSF (200 μM) (*C*). (*D*) Effects of the free radical scavengers vitamin E (10 μM) and NMPG (10 μM) on PDGF-BB-induced vasculogenesis. *$P < 0.05$, significantly different as indicated.

control sample was not impaired. Our data thus demonstrate that the PDGF-BB-mediated vascularization in differentiating ES cells is regulated by the ERK1,2 signalling cascade, which has been previously shown by us to be activated by ROS.[16,19]

4. Discussion

In the present study, the signalling pathways underlying the pro-vasculogenic effects of PDGF-BB in differentiating embryoid bodies derived from ES cells were investigated. PDGF-BB dose-dependently increased the vascularization of embryoid bodies and the mRNA expression of CD31. Interestingly, VE-cadherin mRNA expression was not increased upon PDGF-BB treatment, which corroborates recent data of Rolny *et al.*[10] on VE-cadherin mRNA expression upon treatment of ES cells with PDGF-BB.

PDGF-BB is well known to enhance smooth muscle cell proliferation.[20] There was no increase in the smooth muscle cell marker expression upon PDGF-BB treatment; for α-SMA and caldesmon, we observed a tendency of increased mRNA expression, which, however, did not reach statistical significance. These data are in contrast to the data of Rolny *et al.*[10] who showed a modest but significant upregulation of α-SMA and the pericyte marker RGS-5, whereas—in contrast to the present study—no upregulation of CD31 was observed.

PDGF-BB treatment raised intracellular ROS in embryoid bodies which was abolished in the presence of NADPH oxidase inhibitors. Our studies[17] and others[21] have previously characterized the expression of NOX enzymes in differentiating ES cells and have unravelled the importance of ROS generation for cardiovascular differentiation. ROS have been shown to arise in differentiating ES cells upon electrical field treatment[19] and mechanical strain[16] and to enhance vasculogenesis via VEGF/HIF-1α-dependent pathways that involve upstream MAPK activation. ROS generation upon PDGF treatment is a well-known feature and has been demonstrated to occur in several cell types, including smooth muscle cells.[22,23] In contrast, ROS generation in endothelial cells upon treatment with PDGF-BB has not yet been demonstrated. This may be due to the lack of expression of PDGFR-β in mature endothelial cells, whereas PDGFR-β expression has been demonstrated in haemangioprecursor cells in the yolk sac of mouse embryos and differentiating ES cells[10] as well as under pathological

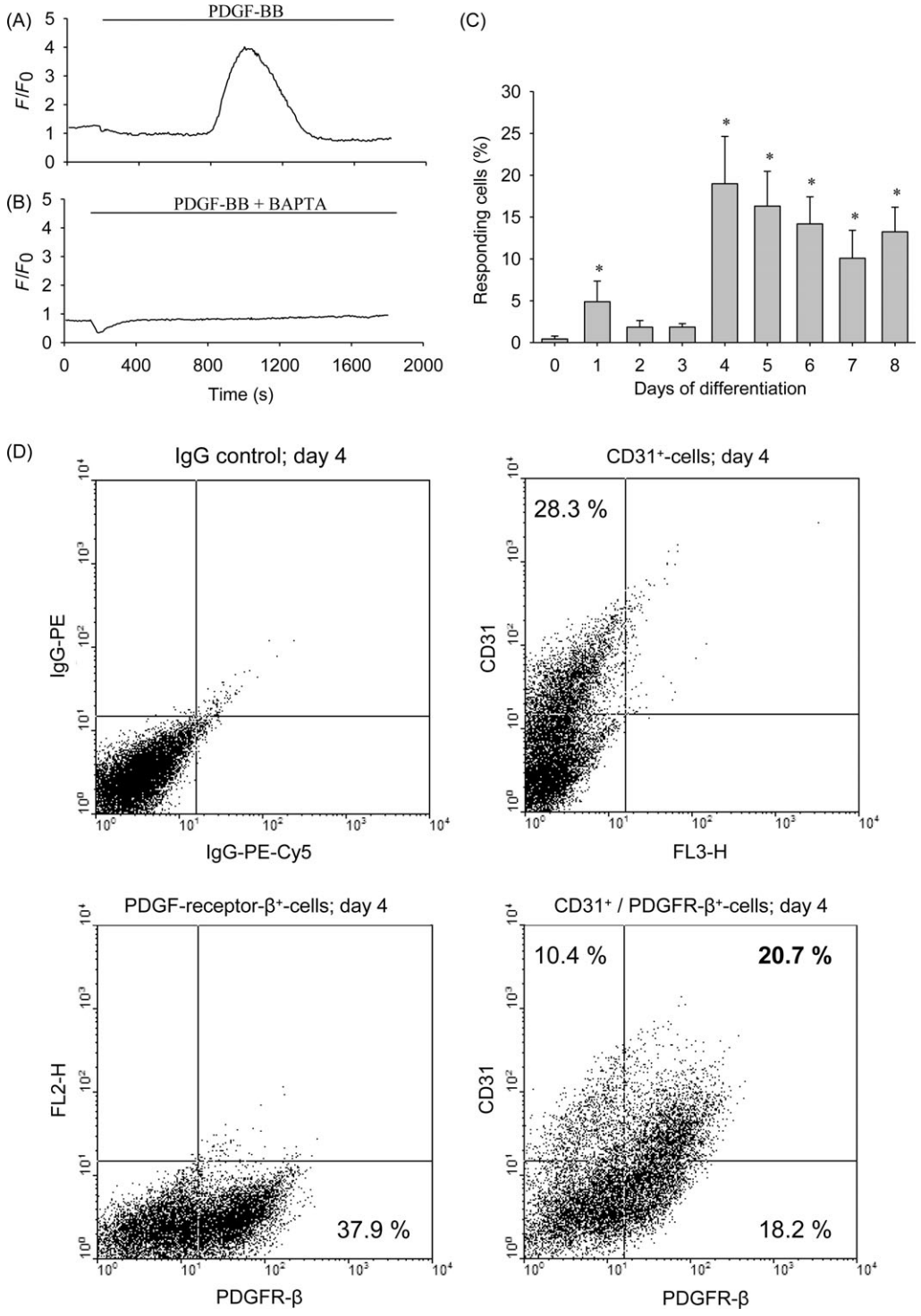

Figure 5 Intracellular Ca^{2+} responses elicited by PDGF-BB in differentiating ES cells and expression of PDGFR-β. (*A* and *B*) Representative traces of intracellular Ca^{2+} in single cells treated with 10 ng/mL PDGF-BB in the absence (*A*) and in the presence (*B*) of the intracellular Ca^{2+} chelator BAPTA/AM (10 μM). PDGF-BB was present in the incubation medium during the time indicated by the horizontal lines. (*C*) Percentage of cells reponding with Ca^{2+} signals in the presence of PDGF-BB (10 ng/mL) in relation to the cell culture time. The number of responding cells is maximum during the time vasculogenesis occurs in embryoid bodies, i.e. between days 4 and 6 of differentiation. (*D*) FACS analysis of CD31 and PDGFR-β expression in cells of enzymatically dissociated 4-day-old embryoid bodies. Upper left, IgG control; upper right, percentage of cells staining positive for CD31; lower left, percentage of cells staining positive for PDGFR-β; lower right, percentage of cells staining positive for CD31 as well as PDGFR-β. $^*P < 0.05$, significantly different from undifferentiated ES cells on day 0 of differentiation.

conditions, e.g. within the immature vasculature of tumours,[7] in the cardiac capillary endothelium during acute and chronic rejection of rat cardiac allografts,[24] and in newly formed blood vessels in an *in vivo* hindlimb ischaemia model.[25] The generation of ROS in embryoid bodies upon treatment with PDGF-BB was preceded by a transient Ca^{2+} response. Ca^{2+} responses occurred very rarely in undifferentiated ES cells and in cells of early days

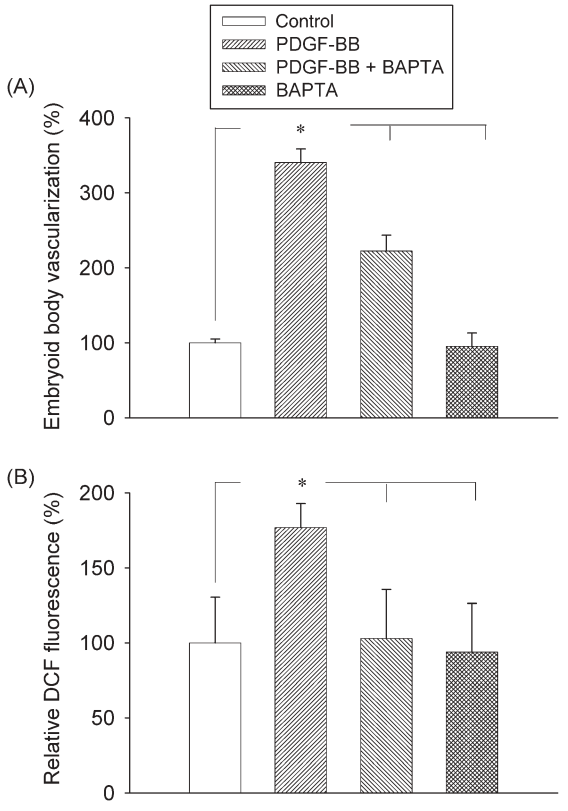

Figure 6 Effects of chelation of intracellular Ca^{2+} on the vascularization of embryoid bodies achieved with PDGF-BB (*A*) and on PDGF-BB-induced ROS generation (*B*). BAPTA/AM (10 μM) was present during the time of incubation with PDGF-BB. In the presence of BAPTA/AM, the stimulation of vascularization of embryoid bodies was significantly inhibited, whereas BAPTA *per se* did not impair vasculogenesis. The increase in ROS generation achieved with PDGF-BB was totally abolished in the presence of BAPTA/AM. $^*P < 0.05$, significantly different as indicated.

of LIF-free cell culture, which suggests absence of PDGFR-β expression during early stages of differentiation. From day 4 on ~20% of the cells responded towards PDGF-BB with a transient Ca^{2+} response, which was in good correlation with the percentage of cells that stained positive for both PDGFR-β and CD31 on day 4 of cell culture. The intracellular Ca^{2+} response was abolished in the presence of the intracellular Ca^{2+} chelator BAPTA, indicating involvement of Ca^{2+} release from intracellular stores. Notably first CD31-positive cell clusters occur in differentiating embryoid bodies on day 5 of cell culture,[26] which closely follows the appearance of Ca^{2+} responses in differentiating ES cells and suggests the involvement of Ca^{2+} signals in the process of endothelial cell commitment. It has been suggested that upon binding of PDGF, the activated PDGF-receptor initiates PLC-γ activity which leads to PIP_2 hydrolysis, resulting in generation of diacylglycerol and IP_3. Subsequently, intracellular levels of Ca^{2+} are elevated as a result of IP_3-mediated Ca^{2+} release from intracellular compartments.[18] Previously it has been shown that the additive effects of the ROS generator and PDGF-BB on rat aortic smooth muscle cells could be inhibited in the presence of the Ca^{2+} chelator EGTA.[27] Direct activation of several NOX isoenzymes, e.g. NOX-5,[28] NOX-2,[29,30] and DUOX2,[31] by intracellular Ca^{2+} signals has been previously evidenced. NOX-5 contains four Ca^{2+} binding sites, and it was demonstrated that Ca^{2+}-induced conformation change of NOX5

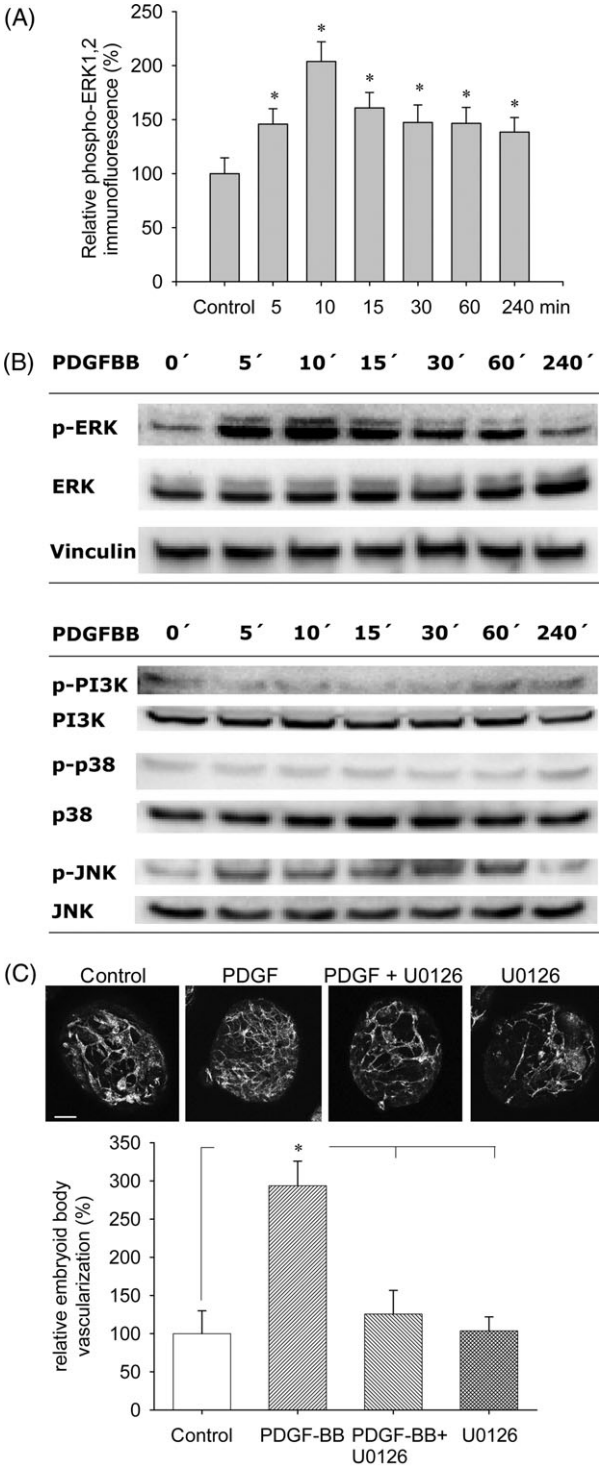

Figure 7 Activation of ERK1,2 upon PDGF-BB treatment of 4-day-old embryoid bodies. (*A*) Time course of ERK1,2 activation following addition of PDGF-BB as investigated by semi-quantitative immunohistochemistry. Maximum activation was achieved after 10 min; after 240 min, ERK1,2 activation remained on an elevated plateau. (*B*) Immunoblots of p-ERK1,2 and ERK1,2 (upper panel), p-PI3K and PI3K, p-p38 and p38, p-JNK and JNK (lower panel). In contrast to ERK1,2, no activation was observed for PI3K and p38 and only low activation was oberved for JNK; no changes in absolute protein levels were observed upon treatment with PDGF-BB. As a loading control, vinculin was used. (*C*) Effects of the MEK1,2 inhibitor U0126 on PDGF-BB-induced vasculogenesis. The upper panel shows embryoid bodies immunostained for CD31 expression. From the left to the right, control, PDGF-BB (10 ng/mL), PDGF-BB+U0126 (10 μM), U0126. In the presence of U0126, PDGF-BB-induced vasculogenesis was completely abolished. $^*P < 0.05$, significantly different as indicated.

N-terminus led to enzyme activation through an intramolecular interaction.[32] In the present study, the increase in ROS generation was totally abolished in the presence of BAPTA, thus indicating that in differentiating ES cells, the activity of NOX-enzymes may be regulated by Ca^{2+} signals. In the present study, the pro-vasculogenic effects of PDGF-BB were blunted in the presence of free radical scavengers and NADPH oxidase inhibitors and consequently in the presence of BAPTA, indicating a signal transduction cascade involving Ca^{2+}-mediated activation of NADPH oxidase. Furthermore, the ERK1,2 MAPK pathway was identified as the signalling pathway involved in the pro-vasculogenic effect of PDGF-BB, whereas activation of PI3K and p38 was absent and only a weak activation was observed for JNK. Activation of ERK1,2 in ROS-mediated vasculogenesis of ES cells has been previously shown by us,[16,19] whereas p38 was not required. In our previous studies, we demonstrated that vasculogenesis in ES cells involved JNK, which was not evident upon treatment with PDGF-BB. Presumably JNK pathways are only active in angiogenesis under acute stress situations like mechanical strain and electrical field treatment but not under conditions of physiological angiogenesis.

The ROS- and Ca^{2+}-mediated signalling pathways elicited by PDGF-BB in differentiating ES cells and enhancing vasculogenesis mimick VEGF-mediated signalling pathways in distinct aspects. Comparably to PDGF, VEGF-mediated signalling pathways have been shown to include transient Ca^{2+} responses,[33] generation of intracellular ROS[34] and ERK1,2 activation.[35] Notably elevation of ROS has been shown to increase expression of HIF-1α and VEGF,[36,37] which suggests that ROS generated through PDGF-BB may induce VEGF gene expression. Indeed, it has been recently shown that inhibition of PDGF-BB signalling downregulated VEGFR-2 expression,[38] whereas upregulation of PDGF-B mRNA and secretion of PDGF-BB were achieved upon stimulation of cells with VEGF,[39] indicating a close interrelation between the two signalling pathways. The interplay between PDGF- and VEGF-mediated signalling pathways is just emerging. Presumably, both PDGF- and VEGF-related receptor signalling pathways are involved in distinct phases of vasculogenesis guiding that use different effector pathways in each and utilize differences in signal levels between cells to guide cell differentiation and migration.[40] Hence, knowledge on the keys of PDGF/VEGF interaction will be of pivotal importance for the understanding of and interference with pathological angiogenesis—but also for the development of strategies for therapeutic pro-angiogenic patient treatment by enhancing angiogenesis guidance in ischaemic diseases.

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