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Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia

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1 We have previously shown that hypoxia makes vascular smooth muscle cells (VSMCs) responsive to placental growth factor (PlGF) through the induction of functional fms-like tyrosine kinase (Flt-1) receptors. The aim of this study was to investigate the molecular mechanisms involved in the PlGF effects on proliferation and contraction of VSMCs previously exposed to hypoxia (3% O_2).

2 In cultured rat VSMCs exposed to hypoxia, PlGF increased the phosphorylation of protein kinase B (Akt), p38 and STAT3; activation of STAT3 was higher than that of other kinases. In agreement with this finding, the proliferation of hypoxia-treated VSMCs in response to PlGF was significantly impaired by the p38 and the phosphatidylinositol 3-kinase inhibitors SB202190 and LY294002, respectively, and was almost completely prevented by AG490, a janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) inhibitor.

3 Since hypoxia was able to reverse the vasorelaxant effect of PlGF into a vasoconstrictor response, the mechanism of this latter effect was also investigated. Significant Flt-1 activity was measured in isolated preparations from rat aorta exposed to hypoxia. Inhibitors of mitogen-activated protein kinase kinase, Akt and STAT3 induced a modest inhibition of the vasoconstrictor response to PlGF, while the p38 inhibitor SB202190 markedly impaired the PlGF-induced contractile response. These effects were selectively mediated by Flt-1 without any involvement of foetal liver kinase-1 receptors.

4 These data are the first evidence that different intracellular pathways activated by Flt-1 receptor in VSMCs are involved in diverse biological effects of PlGF: while mitogen activated protein kinase kinase/extracellular signal regulated $kinase_{1/2}$ and JAK/STAT play a role in VSMC proliferation, p38 is involved in VSMC contraction. These findings may highlight the role of PlGF in vascular pathology.
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Abbreviations: Akt, protein kinase B; $ERK_{1/2}$, extracellular signal regulated $kinase_{1/2}$; Flk-1, foetal liver kinase-1; Flt-1, fms-like tyrosine kinase; HIF, hypoxia-inducible factor; JAK, janus tyrosine kinase; KDR, kinase insert domain protein receptor; MAPK, mitogen activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; STAT, signal transducer and activator of transcription; VEGF-A, vascular endothelial growth factor-A; VSMCs, vascular smooth muscle cells

Introduction

Pathological conditions leading to flow decrease and ischaemia activate multistep processes leading to vessel remodelling, characterised by important structural and functional changes in the vessel wall. Vascular smooth muscle cell (VSMC) proliferation is a key event involved in neo-intimal growth during atherosclerotic plaque formation and restenosis as well as in the arteriogenic process. Arteriogenesis is necessary to limit the size of the area submitted to hypoxic damage and to restore blood flow after the occlusion of a major artery (Buschmann & Schaper, 2000). Hypoxia is a potent regulator of a variety of biological processes, including angiogenesis and vascular contractility (Ladoux & Frelin, 1993; Caudill *et al*., 1998). Many vessel growth factors (Rakugi *et al*., 1990; Katayose *et al*., 1993), as well as procontractile agents responsible for vascular remodelling, are upregulated by hypoxia, as shown in pulmonary hypertension (Raj & Shimoda, 2002). The gene of vascular endothelial growth factor receptor-1 (VEGFR-1 or fms-like tyrosine kinase (Flt-1)) is highly involved in the response to hypoxia since it is activated by an hypoxia-induced factor (HIF)-dependent mechanism (Gerber *et al*., 1997). Flt-1 receptors have been demonstrated to be almost exclusively expressed by endothelial cells and their function seems to be confined to the regulation of VEGF-induced angiogenesis *via* the kinase insert domain protein receptor (KDR) (Park *et al*., 1994). However, it has been recently observed that Flt-1 may also be expressed in VSMCs submitted to mechanical and hypoxic stress (Couper *et al*., 1997; Parenti *et al*., 2002), thus suggesting a potential role of Flt-1 in vascular pathology. Flt-1 is a typical tyrosine

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kinase receptor, and its intracellular pathways, activated by the selective agonist placental growth factor (PlGF), have been extensively identified in endothelial cells. Activation of Flt-1 is followed by the mitogen activated protein kinase (MAPK) cascade, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) activation, which are all responsible for the proliferation as well as for the antiapoptotic signals for endothelium (Landgren *et al*., 1998; Neufeld *et al*., 1999). Flt-1 is also involved in the migration of monocytes (Barleon *et al*., 1996). More recently, it has been demonstrated that Flt-1 activation in smooth muscle cells leads to metalloproteinase activation/expression (Wang & Keiser, 1998). We have previously shown that, following hypoxia, VSMCs proliferate in response to PlGF and that this mitogenic effect is mediated by the ras/raf/extracellular signal regulated kinase (ERK) pathway (Parenti *et al*., 2002). In the same study, it has also been shown that severe hypoxic treatment of rat aorta preparations causes an upregulation of Flt-1, which, in turn, is responsible for a reversal of the relaxant effect of PlGF into a constrictor response (Parenti *et al*., 2002). Owing to the above-cited observations and since little information is available on the intracellular mechanisms linked to Flt-1 receptor activation in non-endothelial cells, the aim of the present study was to assess the intracellular pathways activated by PlGF in VSMCs after hypoxic treatment. The role of the PI3K/Akt, of janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) as well as of the MAPK pathways was assessed in two experimental models, that is, the proliferation of cultured rat VSMCs and the contractile response of endothelium-deprived isolated preparations from rat aorta, exposed to a severe hypoxic treatment.

Methods

Cell culture and cell proliferation study

VSMCs were isolated from the thoracic aorta of male Wistar rats and cultured as previously described (Parenti *et al*., 2001). The obtained cells were cultured in DMEM supplemented with 10% foetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, and were characterised by immunohistochemical assay with an anti-α-actin monoclonal antibody (Sigma), showing that >95% of cells were α-actin positive. VSMC proliferation was quantified by the total cell number as previously reported (Parenti *et al*., 2001). Briefly, 5×10^3 cells were seeded onto 48-multiwell plates and allowed to adhere overnight. Cells were kept in starving conditions (0.1% FCS) for 48 h in normoxic (21% O_2) or in hypoxic conditions (3% O_2). Then, media were removed and replaced with 1% FCS medium containing the test substances. Proliferation was evaluated after 96 h in normoxic conditions. The effect of PlGF was compared with the control condition in 1% FCS medium.

Animals

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats (220–250 g) were purchased from Harlan (S. Pietro AL Natisone, Italy) and killed by cervical dislocation.

Studies on vascular tone

Rings (3–4 mm width) of thoracic rat aorta were mounted in a 10 ml organ bath filled with warmed (37°C) and oxygenated (95% O_2, 5% CO_2) Tyrode solution for isometric measurement, as previously described (Amerini *et al*., 1997). The solution had the following mM composition: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. The preparations were allowed to equilibrate for at least 90 min; during this period, the bath medium was replaced every 15 min. Changes in isometric tension were recorded on a polygraph chart (Battaglia Rangoni, San Giorgio di Piano, Bologna, Italy). After stabilisation, concentration–response curves for NA (0.1–1 μM) were performed and a concentration that induced 50% of the maximum effect was chosen in order to evaluate the effect of test substances. The effect of PlGF was tested in rat aorta rings submitted to a 24-h hypoxic pretreatment, after endothelium deprivation obtained by carefully removing the intimal surface using polyethylene tubing (Amerini *et al*., 1997). Hypoxic treatment was performed by incubating aorta rings in Tyrode solution in a humified incubator with 3% O_2 for 24 h. A 30-min pretreatment with selected antagonists and/or inhibitors was performed before repeating the curve for the drug. Results are expressed as percent of maximal PlGF-induced contraction.

Immunoprecipitation and kinase assay for Flt-1 activity

Aortic rings deprived of endothelium exposed or not exposed to hypoxia were used to measure PlGF-induced contraction. At 3 min after PlGF addition, the preparations were quickly frozen in liquid nitrogen, then homogenised on ice in lysis buffer followed by centrifugation at $14{,}000 \times g$ for 10 min at 4°C. Aliquots of 500 μg of total proteins were used to immunoprecipitate *flt-1* with a polyclonal rabbit IgG antibody (1 μg tube^{-1}, Santa Cruz Biotech., CA, U.S.A.). The immunoprecipitates were washed three times and then used in a kinase assay *in vitro* for Flt-1 activity as reported elsewhere, with some modifications (Dougher & Terman, 1999). The final concentrations of reagents were: 40 mM HEPES pH 7.4, 0.1 mM orthovanadate, 10 mM $MnCl_2$, 100 μg ml^{-1} poly(Glu : Tyr 4 : 1), 25 μM cold ATP and 5 mCi of [γ^{32}P]ATP. The reaction was carried out in 50 μl at room temperature for 30 min. Samples were microfuged for 2 min to pellet the resin, and 25 μl of samples were spotted onto dry P81 paper, washed three times with 0.5% phosphoric acid and then counted by liquid scintillation counting.

Western blot analysis

VSMCs were lysed in buffer followed by centrifugation at $14{,}000 \times g$ for 10 min at 4°C as previously reported (Parenti *et al*., 2002). Aliquots of 50 μg of total proteins were run on 10% SDS–PAGE gels, then transferred to a polyvinylidene difluoride (PVDF) membrane and treated with the anti-phospho STAT3 [Tyr^{705}] (rabbit polyclonal, 1 : 1000), p38 [$pTyr^{1807182}$] (rabbit polyclonal, 1 : 2000), Akt [$pSer^{473}$] (rabbit polyclonal, 1 : 1000) and anti-p-extracellular signal regulated $kinase_{1/2}$ ($ERK_{1/2}$) [Thr^{202}/Tyr^{204}] (mouse monoclonal IgG1,

1 : 2000) antibodies, followed by secondary antibodies (anti-rabbit IgG peroxidase-linked antibody from donkey 1 : 10,000 or anti-mouse IgG1 peroxidase-linked antibody from goat, 1 : 10,000). Immunoreactive proteins were detected by enhanced chemoluminescence (ECL).

RT–PCR analysis

Total RNA from VSMCs was extracted as previously reported (Parenti *et al*., 2001). Aortic fragments isolated from Wistar rats were promptly frozen. Total RNA was extracted using the RNAzol method (Ultraspec RNA, Biotecx) and reverse transcription of 1 μg of total RNA was carried out as previously described (Parenti *et al*., 2002). Foetal liver kinase-1 (Flk-1) and GAPDH mRNAs were amplified with specific primers as follows: Flk-1 sense: 5′-TTg CTT gCT CTC ACg gTT gg-3′; antisense 5′-TgC gTC ATT TCC TCC CTg gg-3′ (116 bp, Schratzberger *et al*., 2000) and GAPDH sense 5′-CTA CTG GCG CTG CCA AGG CTG T-3′; antisense 5′-GCC ATG AGG TCC ACC ACC CTG TTG-3′ (354 bp, Simmons *et al*., 1999). RT–PCR was performed in sequential cycles (28 cycles for GAPDH, 32 cycles for Flk-1) including 30 s denaturation at 94°C, 30 s annealing conditions at 55°C and 30 s extension at 72°C. Amplificates were electrophoresed in 1.5% agarose gel and PCR products highlighted by ethidium bromide.

Materials

PlGF was purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Noradrenaline, indomethacin, wortmannin, poly(Glu : Tyr 4 : 1) and cell culture media and reagents were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Calf serum was purchased from Hyclone (Logan, UT, U.S.A.); Diff-Quik from Merz + Dade AG (Switzerland); LY294002, VEGF-A from Calbiochem-Novabiochem Int. (San Diego, CA, U.S.A.); AG 490, U0126, SB202190 from Tocris Cookson Ltd (Avonmouth, U.K.); anti p-p38 [pTyr$^{180/182}$] was from BioSource Europe SA (Nivelles, Belgium); anti p-Akt [pSer473], anti-p-STAT3 and anti-p-ERK$_{1/2}$ [Thr^{202}/Tyr^{204}] were from Cell Signaling Technology (Frankfurt, Germany); anti-rabbit IgG peroxidase-linked antibody was from Amersham International Biotech (U.K.); anti-mouse IgG1 peroxidase-linked antibody was from Calbiochem-Novabiochem Int. (San Diego, CA, U.S.A.); Akt1 IP-kinase assay kit was from Upstate (Lake Placid, NY, U.S.A.). Acrylamide, TEMED, ammonium persulphate, Coomassie brilliant blue were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). [γ^{32}P]ATP was from NEN (Boston, MA, U.S.A.).

LY294002, AG490, U0126 and SB202190 were dissolved in DMSO and further diluted in DMEM, for cell proliferation assay, and in Tyrode solution, for vascular tone experiments. It is noteworthy that the amount of DMSO present at the highest inhibitor concentration used (0.001, 0.01 and 0.1% for wortmanin, SB202190 and AG40, LY294002, U0126, respectively) did not affect either cell proliferation or vascular tone.

Statistical evaluation

Data are reported as means ± s.e.m. Each experiment was run in duplicate or triplicate. Statistical analysis was performed using Student's *t*-test for unpaired data and analysis of variance followed by the Bonferroni test. A $P<0.05$ was considered significant.

Results

Flt-1 signalling involved in proliferation of cultured VSMCs

VSMCs previously exposed to hypoxia significantly proliferated in response to 10 ng ml^{-1} PlGF (Parenti *et al*., 2002 and Figure 1a). This effect was mediated by Flt-1; it was not attributable to a possible transactivation of Flk-1 receptor as suggested by Autiero *et al*. (2003), since Flk-1 receptor was not detectable in VSMCs. RT–PCR analysis demonstrated the lack of this receptor at the messenger level (Figure 1b), either in cultured VSMCs or in rat aorta samples deprived of endothelium, thus confirming our previous results obtained by immunohistochemical analysis (Parenti *et al*., 2002).

To further investigate the intracellular mechanisms activated by PlGF that were possibly responsible for its mitogenic effects, growth-arrested VSMCs previously exposed to hypoxia were stimulated by 20 ng ml^{-1} PlGF after pretreatment for 1 h with LY294002 (10–30 μM), AG 490 (1–10 μM) and SB202190 (1–10 μM), able to inhibit PI3K, JAK and p38, respectively. All three agents significantly inhibited the proliferative effect of PlGF, as shown in Figure 1, in which the effect of the concentration (10 μM) able to induce a maximal inhibitory

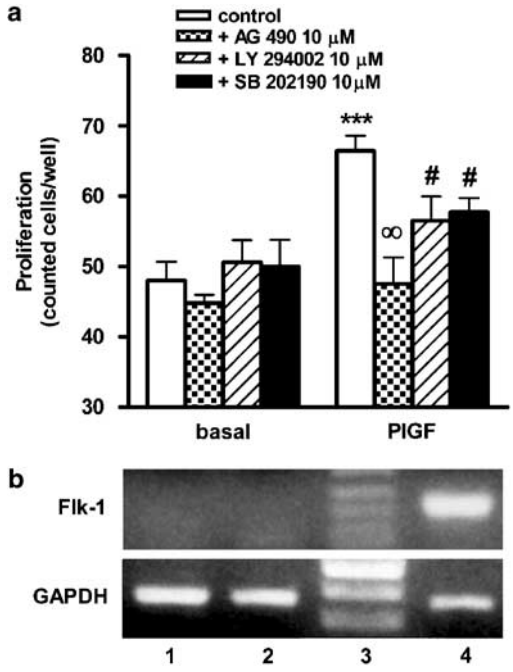

Figure 1 (a) Effect of AG490, SB202190 and LY294002 on the PlGF-induced proliferation of cultured VSMCs. Growth-arrested and hypoxia-treated VSMCs were stimulated with 20 ng ml^{-1} PlGF in the presence and in the absence (control) of the inhibitors. Basal: unstimulated cells in low serum concentration (1%). Means ± s.e.m. of four experiments in duplicate. $^{\#}P<0.05$, $^{\infty}P<0.001$ vs control (PlGF alone); $^{***}P<0.001$ vs unstimulated cells (basal). (b) RT–PCR for mRNA expression in rat VSMCs (1), in endothelium-deprived rat aorta (2) and in rat aorta with endothelium (4); lane 3: marker, DNA ladder.

effect on cell proliferation is shown. However, while SB202190 and LY294002 inhibited cell proliferation by 64±7 and 65±3%, respectively, AG 490 almost fully prevented PlGF-induced VSMC proliferation, displaying the highest inhibitory effect (84.5±1.6% inhibition vs PlGF alone). It is noteworthy that the three inhibitors did not significantly affect basal proliferation and were devoid of any toxic effect, as shown by trypan blue exclusion observations (Table 1). Activation of Akt, STAT3 and p38 was also directly evaluated in VSMCs, either exposed or not exposed to hypoxia, after stimulation with 20 ng ml^{-1} PlGF. Immunoblotting experiments showed that the tyrosine-phosphorylated form of STAT3 increased in a time-dependent manner in response to PlGF only in cells previously exposed for 48 h to hypoxia (Figure 2a). This activation occurred within 5 min and was sustained throughout the whole 30 min treatment period. Akt was also significantly activated within 5 min, only in VSMCs exposed to hypoxia (Figure 2b). P38 was also phosphorylated but only after 5 min stimulation with PlGF in cells pretreated with hypoxia (Figure 2c).

In order to assess the possible crosstalk between $ERK_{1/2}$ and JAK/STAT pathways, the phosphorylation of the above-mentioned kinases was tested in the presence of selective inhibitors. Pretreatment with the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (10 μM) resulted in a significant decrease in $ERK_{1/2}$ phosphorylation in cells stimulated with PlGF; on the other hand, the JAK inhibitor AG490 did not affect $ERK_{1/2}$ phosphorylation (Figure 3a and c). Furthermore, STAT3 phosphorylation in response to PlGF was prevented by the JAK inhibitor AG490 but was increased by the MEK inhibitor U0126 (Figure 3b and c).

Flt-1 signalling involved in the contractile response of VSMCs

The relaxant effect of PlGF (25 ng ml^{-1}) reverted to a contractile response, whose maximal extent was obtained 7 min after PlGF addition and amounted to 152.7±6% of the maximum effect induced by noradrenaline in endothelium-deprived isolated preparations from rat aorta exposed to hypoxia for 24 h (Parenti *et al*., 2002). Since this contractile response was attributable to Flt-1 receptors (Parenti *et al*., 2002), their activity was measured in hypoxia-treated preparations. The preparations were promptly frozen 3 min after the addition of 25 ng ml^{-1} PlGF and were used to measure Flt-1 activity by means of a immunocomplex kinase assay. Flt-1 activity was significantly higher in preparations submitted to hypoxia than in those not exposed to hypoxia (66±6% increase over normoxic condition, $P<0.05$, $n=3$), thus confirming that the contractile response to PlGF was attributable to the activation of Flt-1 receptors. To identify

Table 1 Trypan blue exclusion in VSMCs grown in the presence of the p38- (SB202190), JAK- (AG 490) and PI3K- (LY294002) inhibitors

Drug	*% dead cells*
Control	6.15±2
SB202190	6.65±2.2
LY294002	6.55±1.5
AG490	6.29±1.8

The inhibitors (10 μM) were added to growth-arrested cells and the viability was measured after 4 days in culture. Means±s.e.m., $n=3$.

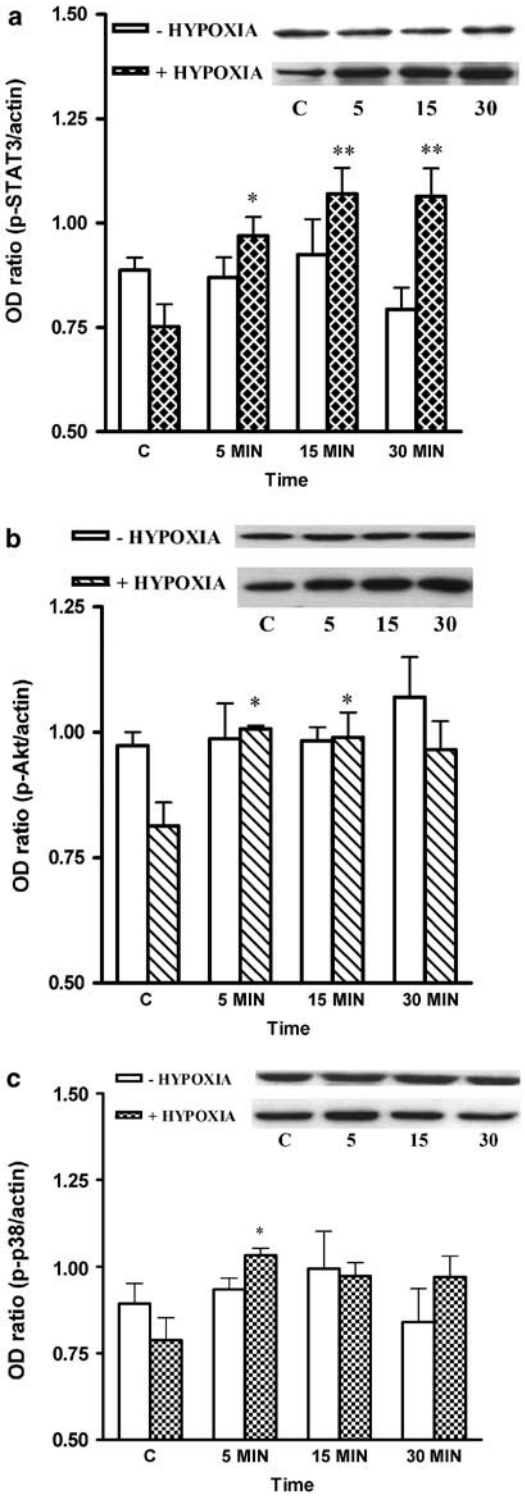

Figure 2 Immunoblots for STAT-3 (a) Akt (b) and p38 (c) phospho-proteins in VSMCs previously exposed to hypoxia and stimulated with 20 ng ml^{-1} PlGF. Data were normalised with actin and are expressed as an immunoreactive band densitometry (OD) ratio between phospho-protein and actin. Means±s.e.m. of 3–5 experiments; *$P<0.05$, **$P<0.01$ vs unstimulated cells exposed to hypoxia.

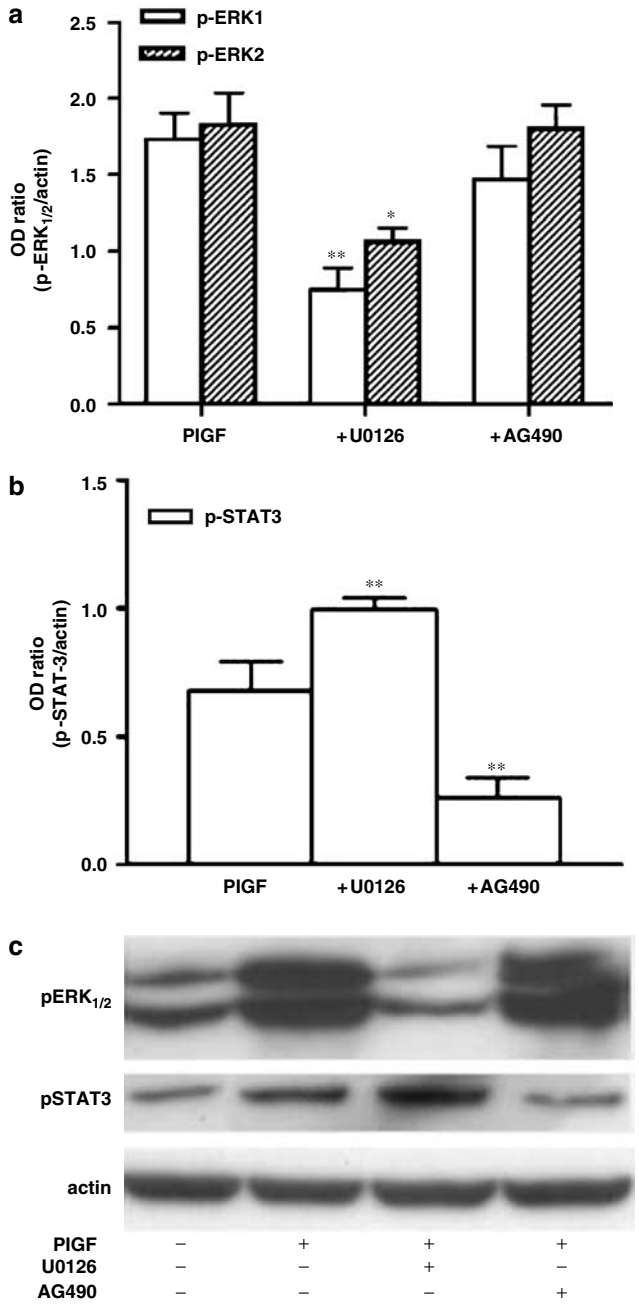

Figure 3 Effect of JAK inhibition on $ERK_{1/2}$ phosphorylation (a) and effect of $MEK/ERK_{1/2}$ inhibition on STAT-3 phosphorylation (b). Cells were preincubated for 48 h in serum-free medium and hypoxia and pretreated with either 10 μM U0126 or 50 μM AG490 for 60 min, and then were treated with 20 ng ml^{-1} PlGF for 15 min. Cell lysates were run on 10% SDS–PAGE gels and then transferred to a polyvinylidene difluoride (PVDF) membrane and treated with the anti-phospho-$ERK_{1/2}$. The membranes were stripped and reblotted with anti-phospho-STAT-3 antibody. Results are expressed as OD ratio using actin as control. Mean $\pm$ s.e.m. of three experiments; $^{**}P<0.01$ vs PlGF alone. (c) Representative blots.

the intracellular pathways involved in vessel contraction induced by Flt-1 stimulation, the effects of specific inhibitors were investigated. Endothelium-deprived preparations exposed to hypoxia were pretreated for 30 min with selective inhibitors of the intracellular pathways, and then the contractile effect of PlGF was measured. These selective inhibitors did not modify the basal tension of the preparations: 980 ± 15.3 mg (untreated) and 956 ± 23.3 (treated with 1 μM Wortmannin); 943.3 ± 53.6 mg (untreated) and 936.7 ± 31.8 mg (treated with 5 μM LY294002); 976.7 ± 12 mg (untreated) and 975.5 ± 11.8 mg (treated with 1 μM AG490); 950.2 ± 18 mg (untreated) and 970 ± 20 mg (treated with 5 μM U0126); 990 ± 25 mg (untreated) and 1010 ± 32.5 mg (treated with 30 nM SB202190).

The contractile response to PlGF was not influenced by a concentration of indomethacin (3 μM) able to block cyclooxygenase activity (data not shown), thus excluding involvement of prostaglandins and thromboxanes from this kind of effect. Two different PI3-kinase inhibitors, wortmannin and LY294002, were used to evaluate the involvement of PI3K. Exposure of the preparations for 30 min to LY294002 (1–10 μM) impaired PlGF-induced vasoconstriction with a maximum response (25.2 ± 3.6% inhibition) obtained with 10 μM (Figure 4a). Similar results were obtained by using the other PI3K inhibitor, wortmannin, which, at the highest concentration used (1 μM), inhibited PlGF-induced vasoconstriction by 20.8 ± 5.2% (four aortic ring preparations from three animals, $P<0.05$). The involvement of the JAK/STAT pathway was tested by exposing the preparations to AG490 (1–30 μM). When this pathway was impaired, the effect of PlGF on the contractile response was inhibited, with a maximum response amounting to 64.2 ± 3.0% of the maximum effect of PlGF obtained with 30 μM AG490 (Figure 4b). The contractile effect of PlGF was also evaluated in the presence of the p38 inhibitor SB202190 (10–1000 nM), and the $MEK_{1/2}$ inhibitor U0126 (1–10 μM). The results of these experiments showed that the $ERK_{1/2}$ pathway was not mainly involved in PlGF-induced VSMC contractile response (Figure 4c). On the contrary, SB202190 concentration-dependently inhibited the contractile response of PlGF, with a maximum effect obtained at the 1 μM concentration that reduced the PlGF effect by 70% (Figure 5).

Discussion and conclusions

Pathological VSMC proliferation and changes in contractile properties are key events involved in the pathophysiology of vascular disorders induced by hypoxia. We have recently reported that, in hypoxic conditions, functional Flt-1 receptors are upregulated in VSMCs, leading to proliferation and contraction in response to VEGF and to the selective Flt-1 agonist, PlGF (Parenti *et al*., 2002). The intracellular pathways involved in the above-mentioned responses were investigated in the present study. The findings obtained demonstrate that the effects on cell proliferation and on contractility induced by PlGF following hypoxia are mediated by two different intracellular pathways. In particular, the stimulation of VSMC proliferation is mainly mediated by JAK/STAT3 pathway activation (present data) and by the $MAPK\text{-}ERK_{1/2}$ cascade (Parenti *et al*., 2002) and, to a lesser extent, by PI3K/Akt pathway and p38. On the other hand, the contractile response is chiefly mediated by p38 activation. Moreover, these PlGF effects are only mediated by activation of Flt-1 receptors without any involvement of Flk-1 transactivation (Autiero *et al*., 2003), since the presence of Flk-1, as both mRNA (present data) and protein (Parenti *et al*., 2002), was not detectable in VSMCs.

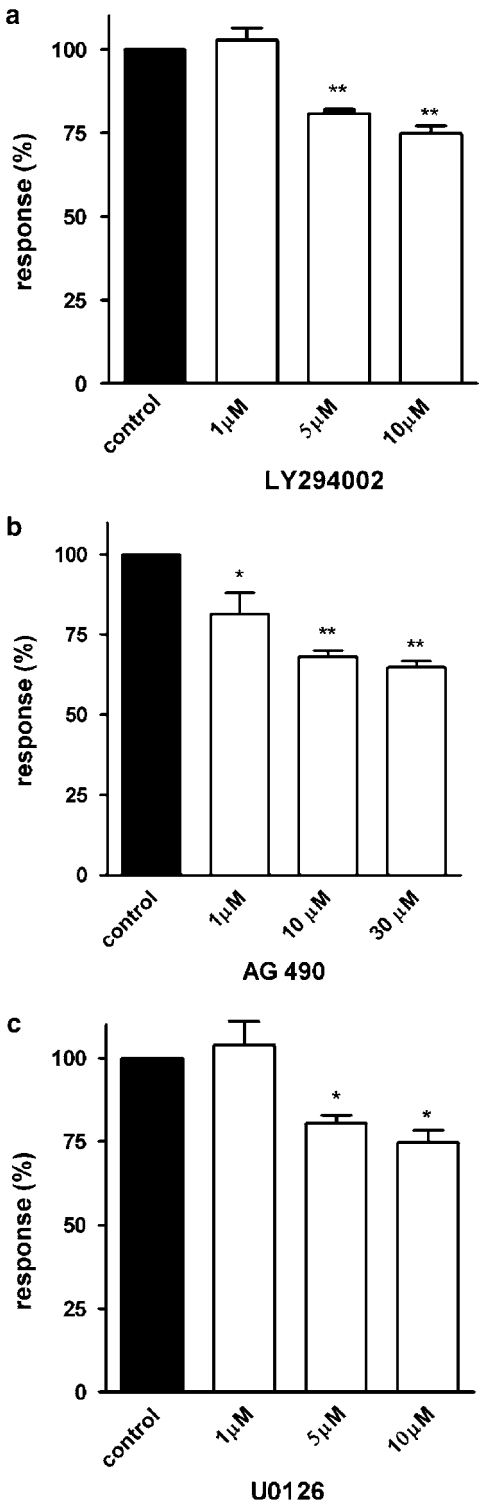

Figure 4 (a) Effect of LY294002 (1–10 μM), (b) AG490 (1–30 μM) and (c) U0126 (1–10 μM) on the PlGF-induced contractile response of vascular preparations exposed to hypoxia. Data are expressed as percent of PlGF-induced contraction (control). Means ± s.e.m. of 4–5 preparations from three to four animals for each concentration point of inhibitor. $*P<0.05$, $**P<0.01$ vs control (PlGF alone).

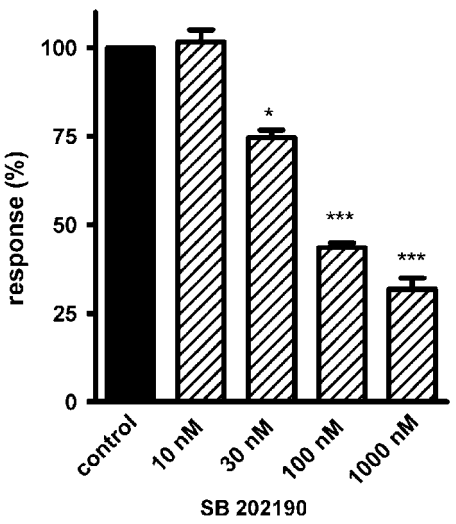

Figure 5 Effect of SB202190 (10–1000 nM) on the contractile response induced by PlGF in preparations exposed to hypoxia. Data are expressed as percent of PlGF-induced contraction (control). Means ± s.e.m. of four preparations from four animals for each concentration point of inhibitor. $**P<0.01$, $***P<0.001$ vs control (PlGF alone).

Hypoxia induces profound effects on blood vessel tone and cell growth. Of the genes induced by hypoxia, Flt-1 has a prominent role. Flt-1 was the first VEGF receptor identified (de Vries *et al*., 1992) and its functions have been extensively investigated in endothelial cells (Neufeld *et al*., 1999; Shibuya 2001; Ferrara *et al*., 2003), monocytes (Barleon *et al*., 1996) and, more recently, in tumour cells (Gerber & Ferrara, 2003) and human eosinophils (Feistritzer *et al*., 2004); nevertheless, the precise function of this receptor is still matter of debate. Either the full-length receptor, or a soluble form *via* an alternative splicing (Shibuya, 2001), was initially proposed as a 'decoy' receptor able to negatively regulate VEGF functions on vascular endothelium (Park *et al*., 1994). However, recent data have shown that the selective Flt-1 agonist PlGF may synergise with VEGF in pathological situations *in vivo*, as evidenced by impaired tumorigenesis and vascular leakage in PlGF−/− mice (Carmeliet *et al*., 2001). Furthermore, the recent findings that Flt-1 may be expressed on VSMCs in conditions of mechanical and hypoxic stress (Parenti *et al*., 2002) suggest that PlGF may contribute to pathological vascular remodelling. With regard to the intracellular pathways activated by Flt-1 stimulation, it is known that the MAPK cascade, as well as PKC and PI3K/Akt activation, are responsible for cell proliferation and antiapoptotic signals in endothelium (Landgren *et al*., 1998; Neufeld *et al*., 1999). The present data, which attempt to clarify the Flt-1-activated pathways in VSMCs, demonstrate that the JAK/STAT pathway is mainly involved in PlGF-induced VSMC proliferation. In fact, STAT3 was significantly and time-dependently activated in VSMCs exposed to a severe hypoxia and stimulated by PlGF, while p38 and Akt were phosphorylated to a lesser extent. Among all the STATs, STAT3 has a potentially relevant role in vascular diseases, since it is activated in response to hypoxia, oxidative stress (Maziere *et al*., 1999), growth factors (Neeli *et al*., 2004) and cytokines (Madamanchi *et al*., 2001; Watanabe *et al*., 2004), and induces VSMC proliferation and neointima formation (Seki *et al*., 2000). Besides the essential role of tyrosine phosphorylation for STATs activation, their serine phosphorylation is implicated in the regulation of transcriptional activity, especially for

STAT1 and STAT3 (Wen *et al*., 1995). Among the many serine kinase(s), $ERK_{1/2}$ has been proposed for this role, since a conserved phosphorylation site was identified within STAT COOH-terminus (Chung *et al*., 1997). Nevertheless, the role of the MAPK cascade on STAT3 activation is still a matter of debate. While a positive regulation of STAT3 activity by the ras/MEK/$ERK_{1/2}$ pathway has been suggested (Ceresa *et al*., 1997; Lim & Cao, 2001), the activation of the MAPK pathway negatively modulates the transcriptional activity of STAT3 in many experimental models (Chung *et al*., 1997; Jain *et al*., 1998). We have attempted to test the possible interactions between the MAPK cascade and the JAK/STAT pathway in VSMCs activated by PlGF. Although ERK phosphorylation was not influenced by the JAK inhibitor AG409, a increase in STAT3 phosphorylation was observed in the presence of the MEK inhibitor U0126, thus suggesting negative modulation of MEK on STAT3 activation.

Extrapolation of these results to pathological conditions *in vivo* is obviously difficult. Nevertheless, our experimental model with primary cultured cells, showing complete prevention of STAT3 phosphorylation and VSMC proliferation in the presence of AG490, seems to suggest a pivotal role for JAK on STAT3-induced cell growth, besides a possible negative modulation by MEK. Moreover, our observations on isolated vascular preparations demonstrate that the contractile response to PlGF, which was observed only in hypoxia-treated preparations, was also mediated by Flt-1 receptor followed by p38 activation. The other kinases studied, that is, PI3K, MEK/$ERK_{1/2}$ and JAK, appeared not to be mainly involved in this kind of effect. P38 was first identified as an inflammatory cytokine-stimulated tyrosine phosphoprotein (Han *et al*., 1994), activated in VSMCs by hypoxia, shear stress (Blaschke *et al*., 2002; Yamawaki *et al*., 2003) and norepinephrine, able to induce vascular smooth muscle hypertrophy, contraction, and cell migration (Kalyankrishna & Malik, 2003). Activation of p38 has also been detected in myocytes in response to ischaemia/reperfusion oxidative stress (Cicconi *et al*., 2003). The present data demonstrated a role for p38 in vessel constriction, which may be involved in the redistribution of collateral circulation aimed at rescue from severe hypoxia following flow insufficiency (Sambuceti *et al*., 1997).

In conclusion, our findings demonstrate that a phenotypical change occurs in VSMCs in response to hypoxia, which makes them able to proliferate and contract in response to PlGF *via* Flt-1 activation. The findings also represent the first demonstration that two different intracellular pathways are involved in these effects: MAPK/$ERK_{1/2}$ and JAK/STAT mediated VSMC proliferation, while p38 is involved in VSMC contractile response.

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