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# Regulation of Raf by Akt Controls Growth and Differentiation in Vascular Smooth Muscle Cells*

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**The stimulation of platelet-derived growth factor (PDGF) receptors shifts vascular smooth muscle (VSM) cells toward a more proliferative phenotype. Thrombin activates the same signaling cascades in VSM cells, namely the Ras/Raf/MEK/ERK and the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathways. Nonetheless, thrombin was not mitogenic, but rather increased the expression of the smooth muscle-specific myosin heavy chain (SM-MHC) indicative of an *in vitro* re-differentiation of VSM cells. A more detailed analysis of the temporal pattern and relative signal intensities revealed marked differences. The strong and biphasic phosphorylation of ERK1/2 in response to thrombin correlated with its ability to increase the activity of the SM-MHC promoter whereas Akt was only partially and transiently phosphorylated. By contrast, PDGF, a potent mitogen in VSM cells, induced a short-lived ERK1/2 phosphorylation but a complete and sustained phosphorylation of Akt. The phosphorylated form of Akt physically interacted with Raf. Moreover, Akt phosphorylated Raf at Ser$^{259}$, resulting in a reduced Raf kinase activity and a termination of MEK and ERK1/2 phosphorylation. Disruption of the PI 3-kinase signaling prevented the PDGF-induced Akt and Raf-Ser$^{259}$ phosphorylation. Under these conditions, PDGF elicited a more sustained MEK and ERK phosphorylation and increased SM-MHC promoter activity. Consistently, in cells that express dominant negative Akt, PDGF increased SM-MHC promoter activity. Furthermore, expression of constitutively active Akt blocked the thrombin-stimulated SM-MHC promoter activity. Thus, we present evidence that the balance and cross-regulation between the PI 3-kinase/Akt and Ras/Raf/MEK signaling cascades determine the temporal pattern of ERK1/2 phosphorylation and may thereby guide the phenotypic modulation of vascular smooth muscle cells.**

Vascular smooth muscle cells determine blood pressure and flow-through modulation of the vascular tone. The contractility depends on the expression of proteins such as smooth muscle $\alpha$-actin and smooth muscle myosin, and their expression levels vary depending on developmental and/or differentiation stage. During progression of vascular diseases or vascular injury following balloon dilatation, the release of growth factors such as PDGF,[1] epidermal growth factor, or IGF has been shown to increase the smooth muscle cell proliferation and migration (1–3). This de-differentiation is characterized by a decreased expression of contractile proteins.

Following ligand binding, tyrosine kinase receptors undergo dimerization which allows transphosphorylation at multiple tyrosine residues. The intracellular signal transduction involves direct interaction of effector molecules via specific domains, *e.g.* Src homology 2 domains and phosphotyrosine-binding domains. More than 10 different Src homology 2 domain-containing molecules have been shown to bind to different autophosphorylation sites in the PDGF receptors, including signal transduction molecules with enzymatic activity like phosphatidylinositol 3-kinases (PI 3-kinases), phospholipases C$\gamma$, or Src as well as adaptor molecules such as Grb2 and Shc (4). Binding of Grb2/Sos or Shc in turn activates the small GTP-binding protein Ras which couples to the Raf/MEK/ERK cascade. The cellular response of receptor tyrosine kinase signaling is influenced by the strength and the duration of ERK1/2 phosphorylation. Depending on the cellular context, either proliferation or differentiation may result (5). Other signaling cascades initiated by PDGF receptors and their potential cross-talk is currently under extensive investigation. Phosphorylated tyrosine residues (Tyr$^{740}$ and Tyr$^{751}$) on the PDGF $\beta$-receptor recruit the PI 3-kinases $\alpha$ and $\beta$ to the plasma membrane via docking of the common p85 regulatory subunit (6, 7). Upon activation, the lipid kinase activity of PI 3-kinases catalyzes the formation of PI(3,4,5)-$P_3$, a well defined plasma membrane anchor for the pleckstrin homology domains of 3-phosphoinositide-dependent kinase I and protein kinase B/Akt (8). The plasma membrane recruitment exposes Akt to subsequent activation by 3-phosphoinositide-dependent kinase I and related kinases that phosphorylate Akt at Thr$^{308}$ and Ser$^{473}$ (9). Akt is a major participant in growth factor-mediated transcription and promotes cell survival by inhibiting apoptosis. These processes appear to involve phosphorylation and inactivation of several targets including Bad (10), forkhead transcription factors (11), and caspase-9 (12). Recent reports by Rommel *et al.* (13) and Zimmerman and Moelling (14) demonstrated that Akt negatively regulates the Ras/Raf/MEK/ERK

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; CAT, chloramphenicol acetyltransferase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, ERK kinase; PI 3-kinase, phosphatidylinositol 3-kinase; SM-$\alpha$-actin, smooth muscle $\alpha$-actin; SM-MHC, smooth muscle myosin heavy chain; VSM, vascular smooth muscle; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; QM, quiescent medium; CM, complete medium; NGF, nerve growth factor; PLC, phospholipase C; IGF, insulin-like growth factor.

pathway via phosphorylation and inactivation of Raf at $Ser^{259}$.

Both PDGF and thrombin receptors qualitatively engage phospholipases C, ERKs, and PI 3-kinases. Nonetheless, in VSM cells, these agonists exert virtually opposite effects regarding the phenotypic modulation. Whereas thrombin via protease-activated receptors and $G\beta\gamma$ released from activated $G_i$ proteins up-regulates the expression of contractile proteins, PDGF treatment exerted no differentiating effect. Vice versa, thrombin stimulation was without significant mitogenic potential, while PDGF almost reconstituted the proliferative effect of serum. To evaluate the contribution of the MAP kinase and PI 3-kinase pathways to the phenotypic modulation of VSM cells, we studied the coupling of PDGF and thrombin receptors to the Ras/Raf/MEK/ERK and the PI 3-kinase/Akt cascades and their cross-regulation. Our results demonstrate that PDGF and thrombin activate both pathways but exerted substantial differences in signal intensity and their kinetic patterns. Biochemical analysis revealed an interaction between Akt and Raf in PDGF-stimulated VSM cells that modulates the late-phase ERK1/2 phosphorylation. Abrogation of the PI 3-kinase/Akt signaling changed the PDGF-induced proliferative response in VSM cells toward enhanced expression of contractile proteins.

## EXPERIMENTAL PROCEDURES

*Materials*—Culture media and trypsin were purchased from Life Technologies. Fetal calf serum and phosphate-buffered saline were obtained from Biochrom. Radiochemicals were from PerkinElmer Life Sciences. The anti-Raf monoclonal antibody was purchased from Transduction Laboratories. Unless otherwise stated, all other antibodies were from New England Biolabs. LY294002, wortmannin, recombinant growth factors PDGF-BB, IGF-I, and epidermal growth factor were obtained from Calbiochem, and recombinant GST-MEK-$His_6$ was from Upstate Biotechnology. All other reagents were obtained from Sigma.

*Cell Culture, Transient Transfections, and Reporter Assays*—Primary cultures of VSM cells from newborn rats were established as previously described (15). Cells were grown in minimal essential medium supplemented with 10% fetal calf serum (complete medium, CM), 2% tryptose phosphate broth, penicillin (50 units/ml), and streptomycin (50 units/ml). In all experiments, cells from passages 10–15 were used. Growth arrest was induced in a serum-free quiescent medium (QM) containing 1% (w/v) bovine serum albumin and 4 mg/ml transferrin instead of serum. Prior to agonist application, cells were maintained in QM for 48–72 h.

The transcriptional regulation of SM-1/SM-2 was assessed with a chloramphenicol acetyltransferase (CAT) reporter gene expressed under the control of the myosin heavy chain promoter (nucleotides −1346 to +25, pCAT-1346) as described (16). For transient transfection assays, cells were seeded into 6-well plates at a density of $7.5 \times 10^4$ cells/well (60–80% confluency) and growth arrested in QM for 48 h prior to transfection. Transient transfections were performed in triplicates with 1 $\mu$g of plasmid DNA and 10 $\mu$l/well Superfect transfection reagent (Qiagen) for 5 h. After 36–48 h, cell lysates were prepared using the CAT Enzyme Assay System (Promega). CAT activities were normalized to the protein concentration of each sample as measured by the BCA assay. Transfection of a promoterless CAT construct served as a baseline indicator, allowing all other promoter constructs to be expressed relative to promoterless activity.

*Immunostaining*—VSM cells were grown to confluency on Nunc Chamber Slides (Nalge Nunc International). After fixation in 1% formaline in phosphate-buffered saline, smooth muscle $\alpha$-actin was detected by using a monoclonal primary antibody (1:150; Sigma) and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:40, Dianova). Representative visual fields were photographed in an epifluorescence microscope (Nikon Diaphot) applying a fluorescein isothiocyanate filter set (Chroma).

*Immunoblotting Procedures*—VSM cells were directly lysed in Laemmli buffer containing 10 mM dithiothreitol. Proteins were separated on polyacrylamide gels and electroblotted to nitrocellulose membranes. Akt, ERK1/2, MEK, or Raf were separated on 10% gels and probed with affinity purified polyclonal anti-phospho-Akt, phospho-ERK1/2, phospho-MEK, and phospho-Raf or with anti-Akt, -ERK1/2, MEK (New England Biolabs), and -Raf antibodies (Transduction Laboratories) to confirm equal loading of the gels. Primary antibodies were detected with a horseradish peroxidase-coupled secondary antibody (1:2000, New England Biolabs) using a chemiluminescence substrate (Lumiglo, New England Biolabs).

*RNase Protection Assay*—RNA isolation, generation of DNA templates, and hybridization conditions were described previously (17). The Maxiscript and RPA II kits from Ambion were used for RNase protection assays. In brief, 10 $\mu$g of total RNA was hybridized with radiolabeled probes overnight at 42 °C. Non-hybridized fragments were digested with RNase A/T1. The remaining protected fragments were separated by denaturing (8% urea) polyacrylamide gel electrophoresis and exposed to Amersham Hyperfilm at −80 °C for 2–24 h. Bands were excised and counted in a liquid scintillation counter. Equal loading was controlled by hybridization with a rat glutaraldehyde-3-phosphate dehydrogenase probe.

*Raf Kinase Assay*—VSM cells were serum starved for 48 h in serum-free medium. After stimulation, cells were lysed in RIPA buffer (14), and Raf protein was immunoprecipitated with an anti-Raf monoclonal antibody (Transduction Laboratories) as described previously (14). *In vitro* kinase assays were performed by incubating the immunocomplexes in 30 $\mu$l of kinase buffer containing 1 $\mu$g of recombinant GST-MEK-$His_6$ (Upstate Biotechnology) and 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP in kinase buffer for 30 min at 30 °C. Proteins were separated by SDS-PAGE, and their phosphorylation was visualized and quantified with a phosphorimaging system (Fuji Bas-1500).

*Immunoprecipitation*—VSM cells were lysed in a 0.25% Nonidet P-40 containing lysis buffer as described previously (14). Cleared lysates (350 $\mu$g of protein in 800 $\mu$l) were immunoprecipitated overnight at 4 °C with 2 $\mu$g of monoclonal anti-Raf-1 antibodies (Transduction Laboratories) coupled to suspended Protein A-coupled Sepharose beads (Sigma). The pelleted beads were washed three times in 400 $\mu$l of lysis buffer. Immunoprecipitates were boiled in SDS-Laemmli buffer and subjected to Western blot analysis with anti-Raf (Transduction Laboratories), anti-Akt and anti-phospho-Akt antibodies (New England Biolabs).

## RESULTS

*Mitogenic Signaling of PDGF Fails to Up-regulate the Expression of Contractile Proteins in VSM Cells*—We have recently demonstrated that serum, in addition to its mitogenic properties, increases the expression of contractile proteins in neonatal rat vascular smooth muscle (VSM) cells (18). To evaluate the proliferative effects of serum, PDGF, and thrombin, VSM cells were cultured in serum-free QM supplemented with the respective agonist. The initial cell counts were assessed at the beginning of the experiment (day 0, Fig. 1*A*) and every following day. To account for potential degradation of the agonists, media were replaced every day. Whereas cell counts remained almost constant in QM, doubling rates in the presence of serum were about 1.5 days. Unlike thrombin (1 unit/ml), PDGF-BB (10 ng/ml) was a powerful mitogen that reconstituted about 80% of the serum-mediated cell proliferation (Fig. 1*A*).

The abundant expression of the contractile protein SM-$\alpha$-actin in VSM cells cultured in serum-containing medium (Fig. 1*B*), however, was not maintained when serum was replaced by PDGF (10 ng/ml; Fig. 1*C*). The quantitative analysis of SM-$\alpha$-actin steady-state expression applying RNase protection assays confirmed that SM-$\alpha$-actin transcripts are highly abundant in VSM cells maintained in serum-containing CM compared with serum-starved (QM) controls. Re-exposure to serum increased the SM-$\alpha$-actin steady-state expression within 24 h by 15-fold, whereas PDGF failed to significantly up-regulate the SM-$\alpha$-actin expression within up to 3 days (Fig. 1, *D* and *E*).

*PDGF Induces a Transient Phosphorylation of ERK1/2 but a Sustained Phosphorylation of Akt*—To define signaling pathways involved in PDGF-induced mitogenesis, we analyzed the activation of ERK1/2 and Akt, a downstream effector of the PDGF-induced PI 3-kinase signaling. Addition of PDGF (10 ng/ml) to serum-starved VSM cells led to a complete ERK1/2 phosphorylation which peaked within 5–10 min and returned to baseline levels at 30–60 min (a representative example of at least three independent experiments showing similar results is

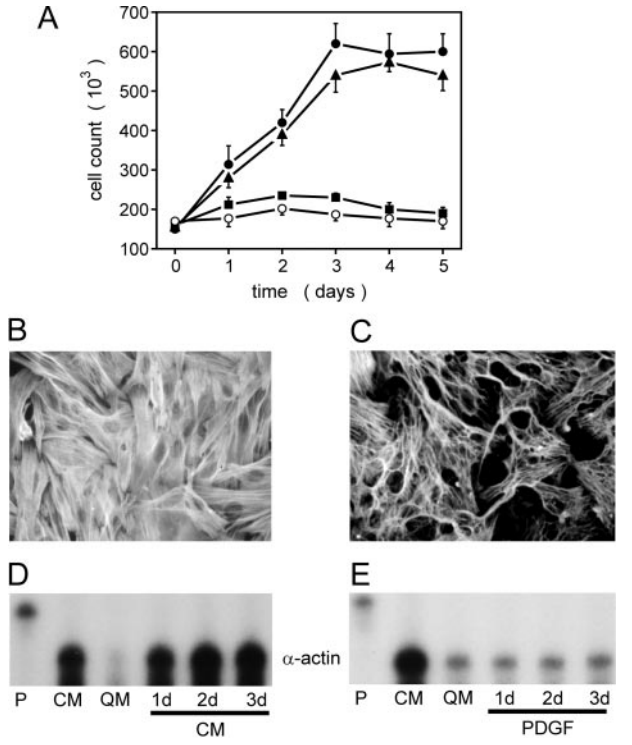


FIG. 1. **Effects of serum and PDGF on vascular smooth muscle cell growth and phenotype.** *A,* rat neonatal VSM cells were maintained in serum-free QM (*open circles*) and were then re-exposed to either serum (*closed circles*) or QM supplemented with PDGF-BB (10 ng/ml, *closed triangles*) or thrombin (1 unit/ml, *closed squares*) for the indicated times. Cells were counted every day in a Neubauer counting chamber. The depicted mean ± S.E. were calculated from at least eight separate counts. *B* and *C,* immunofluorescence analysis of smooth muscle-specific α-actin expression in VSM cells maintained either in serum containing CM (*B*) or in QM containing 10 ng/ml PDGF-BB (*C*). The pictures are representatives of three independent experiments showing similar results. *D* and *E,* RNase protection assay of smooth muscle-specific α-actin steady-state mRNA expression in VSM cells. The cells were maintained in CM, starved in QM for 48 h, and then either re-exposed to CM (*D*) or stimulated with PDGF-BB (10 ng/ml; *E*) for the indicated number of days. The protected fragments of the full-length probe (*P*) correspond to the expected size of 191 nucleotides. Each lane was loaded with 10 μg of total RNA, and hybridization with a glutaraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe confirmed the equal loading (data not shown).

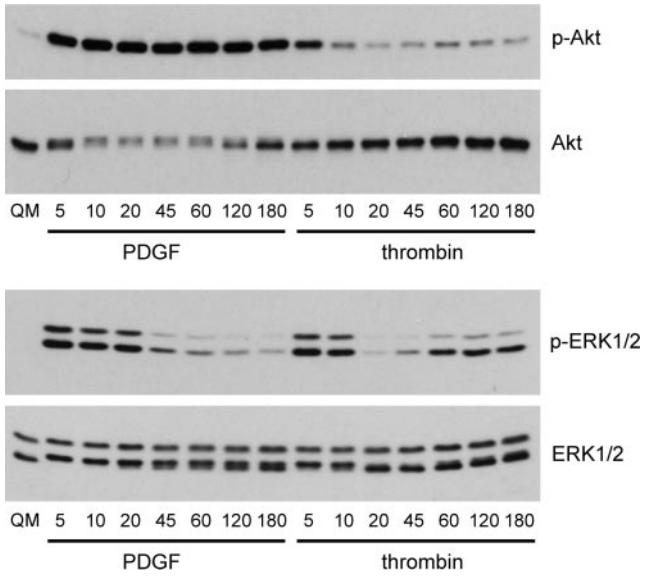


FIG. 2. **Time course of PDGF- and thrombin-mediated phosphorylation of Akt and ERK1/2.** Serum-starved VSM cells were stimulated with 10 ng/ml PDGF-BB or 1 unit/ml thrombin for the indicated times (in minutes). Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted. Activated Akt and ERK1/2 were detected with phospho-S473-specific anti-Akt (*p-Akt*) and anti-phospho-ERK1/2 (*p-ERK1/2*) antisera, respectively. Aliquots of the same lysates were probed with antibodies detecting total Akt and ERK1/2 to demonstrate equal loading and phosphorylation-induced mobility shifts. The experiments shown are representative of three independent experiments with similar results. The reduced Akt signal intensities after stimulation with PDGF presumably reflect a preferential recognition of the unphosphorylated epitope by antibodies raised against amino acids 466–479 of Akt. Note the correlation between the almost complete mobility shift of Akt, and the reduction of the total Akt signal that coincides with the appearance of a phospho-S473-Akt signal.

shown in Fig. 2). Equivalent results were obtained when lysates were probed for activated MEK applying phospho-specific anti-MEK antibodies (data not shown). Probing the same cell lysates with phospho-S473-Akt antibodies revealed a strong PDGF-induced Akt phosphorylation. Of note, antibodies raised against the amino acids 466–479 of Akt preferentially recognized the unphosphorylated state and consistently showed weaker signals when their epitope was phosphorylated at $Ser^{473}$. The almost complete mobility shift of Akt in response to PDGF stimulation correlates with the reduction of anti-Akt signal intensities and gives a means for a semi-quantitative analysis of the phosphorylation state of Akt. In between 10 min and 1 h, the PDGF-induced Akt phosphorylation was almost complete ($n = 12$) and gradually declined within the following 2 h (Fig. 2). Thus, the phosphorylation of ERK1/2 appeared to decline when Akt is activated.

On the other hand, thrombin stimulation resulted in similar early phosphorylation of ERK1/2, but additionally induced a delayed second-phase ERK1/2 phosphorylation. Regarding Akt, thrombin exerted only a weak and transient phosphorylation as compared with the PDGF-stimulated samples that were processed on the same blot (Fig. 2). To estimate the phosphorylated, slower migrating fraction of ERK1/2 and to ensure equal loading of the lanes, blots were reprobed with antibodies detecting total ERK1/2. A comparable extent and kinetic pattern of Akt- and ERK1/2 phosphorylation was achieved when VSM cells were challenged with IGF-I (10 ng/ml). The addition of epidermal growth factor (10 and 100 ng/ml), however, led to a more transient phosphorylation of Akt (data not shown).

Because cross-regulation of the PI 3-kinase/Akt and Ras/Raf/MEK/ERK cascades has been shown to influence proliferation or differentiation of myoblasts and HEK 293 cells by an Akt-dependent association and phosphorylation of Raf (13, 14), we analyzed whether Raf and Akt physically interact in VSM cells. Serum-starved VSM cells were stimulated with PDGF for 15 and 60 min, and Raf was immunoprecipitated from cell lysates applying anti-Raf-1 antibodies. Western blotting of the immunoprecipitates with anti-Akt antibodies revealed an increased interaction between Akt and Raf after 15 and 60 min of PDGF-BB stimulation (Fig. 3). The relative weak signals may be due to a specific interaction of the $Ser^{473}$-phosphorylated form of Akt that is poorly recognized by the antibody. The anti-Raf-1 immunoprecipitates was, therefore, repeated and probed with anti-phospho-Akt antibodies. Indeed, after PDGF treatment, $Ser^{473}$-phosphorylated Akt could be co-immunoprecipitated with anti-Raf-1 antibodies (Fig. 3). Thus, as a consequence of PDGF receptor signaling, the PI 3-kinase/Akt and Raf/MEK/ERK cascades interact at the level of Akt and Raf. The functional consequence of this interaction was therefore investigated.

*Akt-Raf Cross-talk Suppresses the Raf Kinase Activity and Phosphorylation of MEK and ERK1/2*—The PDGF-induced Akt phosphorylation is most likely due to PI 3-kinases that are docked and activated by the tyrosine-phosphorylated receptor.

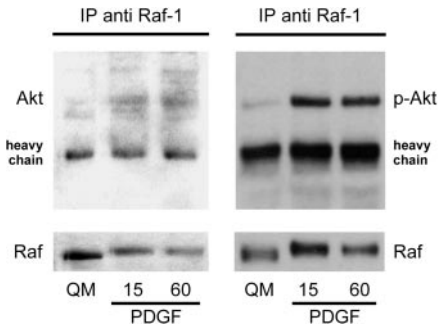


FIG. 3. **PDGF-induced association of phospho-Akt and Raf-1.** Whole cell lysates were prepared from VSM cells maintained in serum-free medium (*QM*) or stimulated with PDGF (10 ng/ml) for the indicated time (in minutes). Lysates were subjected to immunoprecipitation with an anti-Raf-1 antibody (2 $\mu$g). Immunoprecipitates were recovered with protein A-coupled Sepharose beads, washed, and separated by 10% SDS-PAGE. The co-precipitated Akt was detected either with anti-Akt- or anti-phospho-S473-Akt antibodies. Note the reduced affinity of anti-Akt to its phosphorylated epitope amino acids 466–479 as shown in Fig. 2. The recovery of immunoprecipitated Raf is shown in the *lower panels* by probing blots with anti-Raf antibodies. The mobility shift following PDGF stimulation represents the phosphorylation of Raf.

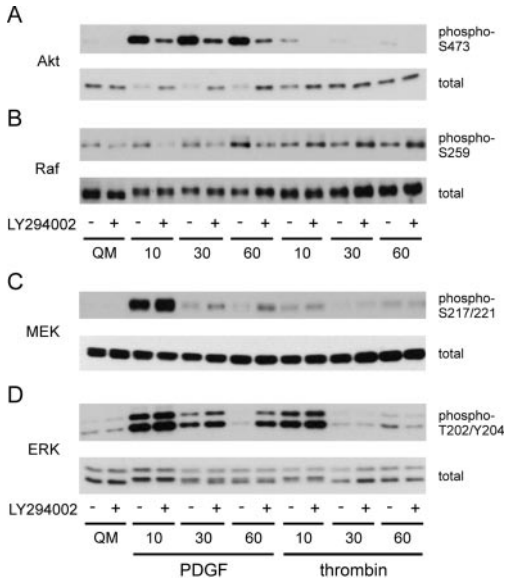


FIG. 4. **Effect of LY294002 on the phosphorylation pattern of Akt, Raf, MEK, and ERK in PDGF- and thrombin-stimulated cells.** VSM cells were treated with LY294002 (20 $\mu$M, *LY*) or its solvent as indicated. After 30 min, cells were stimulated with PDGF-BB (10 ng/ml) or thrombin (1 unit/ml) for the indicated times (in min). For each time point, aliquots of cell lysates were separated on SDS-PAGE and probed for the phosphorylated forms of: *A,* Akt (*phospho-S473*); *B,* Raf (*phospho-S259*); *C,* MEK (*phospho-S217/221*); or *D,* ERK1/2 (*phospho-T202/Y204*). Equal loading and mobility shifts are demonstrated with antibodies detecting the respective total proteins. Note that PDGF- and thrombin-treated samples were loaded on the same gel allowing a direct comparison of signal intensities. The experiment shown is representative of two experiments showing similar results.

To analyze whether the interaction between phospho-Akt and Raf affects the phosphorylation-state of Raf, MEK, and ERK in living VSM cells, we studied the temporal phosphorylation pattern of these molecules in the absence and presence of the PI 3-kinase inhibitor LY294002 (20 $\mu$M). Aliquots of cell lysates were first probed with phospho-S473-Akt and Akt antibodies demonstrating a more than 80% reduction of the PDGF-induced Akt phosphorylation by LY294002 (Fig. 4*A*). Consistent with the findings shown in Fig. 2, thrombin induced a weak Akt phosphorylation at the 10-min time point that was sensitive to the PI 3-kinase inhibitor. At later time points, Akt phosphorylation was not detectable irrespective of the absence or presence of LY294002. A second set of aliquots from the same cell lysates were probed for phospho-S259-Raf and total Raf. After 10, 30, and, most strikingly, after 60 min of PDGF stimulation, the $Ser^{259}$ phosphorylation of Raf was less intense in samples from VSM cells that were pretreated with LY294002 compared with controls without pretreatment. Since it is known that phospho-$Ser^{259}$ serves a docking site for the inhibitory 14-3-3 protein, PI 3-kinase/Akt signaling may thereby reduce Raf kinase activity. By contrast, the thrombin induced moderate increase in $Ser^{259}$ phosphorylation of Raf was further increased in the presence of the PI 3-kinase inhibitor (Fig. 4*B*). At the level of MEK, the PDGF-induced phosphorylation was prolonged in the presence of LY294002, particularly after 60 min, suggesting an altered Raf kinase activity. The enhanced late-phase MEK phosphorylation was of importance since the resulting ERK1/2 phosphorylation was comparable to the peak signals induced by thrombin (Fig. 4, *C* and *D*). Similar results were obtained in a second experiment and in two additional experiments applying wortmannin (100 nM) instead of LY294002 (data not shown). The marked effect of PI 3-kinase inhibition on ERK1/2 and MEK phosphorylation in conjunction with an increased $Ser^{259}$ phosphorylation of Raf points to a regulatory role of PI 3-kinase/Akt on the Raf kinase activity.

We therefore determined the *in vitro* Raf kinase activity by co-incubating immunoprecipitated Raf protein and recombinant GST-MEK in the presence of [$\gamma$-$^{32}$P]ATP. The formed [$^{32}$P]GST-MEK was separated by SDS-PAGE, blotted, and visualized by autoradiography (Fig. 5*A*). The recovery of Raf was assessed by immunoblot analysis of the precipitates (Fig. 5*A*). In the absence of LY294002, the PDGF-induced Raf activity increased about 2-fold ($n$ = three independent experiments) as compared with unstimulated cells at 10 min, 1.4-fold at 30 min, and returned to baseline at 60 min (Fig. 5*B*). In contrast, in the presence of LY294002, Raf activity was about 1.5-fold at 3–10 min, but further increased to 1.7-fold at 30 min and 1.9-fold at 45–60 min ($n$ = 6). Thus, the PI 3-kinase/Akt pathway attenuates the Raf kinase activity and the resulting phosphorylations of MEK and ERK in PDGF-stimulated VSM cells.

The role of PI 3-kinase signaling on the activity of the PDGF and thrombin mediated activity of the Ras/Raf/MEK/ERK cascade was further analyzed by monitoring the extended time course of ERK1/2 phosphorylation in the presence of different concentrations of LY294002. The pretreatment of VSM cells with 20 or 50 $\mu$M LY294002 led to a slightly delayed but long-lasting ERK phosphorylation as compared with solvent-pretreated cells (Fig. 6*A*). Similar alterations in ERK1/2 kinetics were obtained when VSM cells were pretreated with 100 nM wortmannin (data not shown). Thus, by inhibiting PI 3-kinases, the PDGF-induced, short-lived ERK1/2 phosphorylation kinetic was converted into a sustained ERK1/2 activity which is almost comparable to the kinetic of ERK1/2 phosphorylation in response to thrombin (0.1 unit/ml) treatment (Fig. 6*B*). At 50 $\mu$M concentrations, LY294002 diminished the thrombin-induced ERK1/2 phosphorylation, an effect that is consistent with the observed PI 3-kinase-dependent increase in $Ser^{259}$ phosphorylation of Raf (Fig. 4*B*).

*Inhibition of PI 3-Kinases Results in a PDGF-induced Differentiation of VSM Cells*—Considering that a sustained ERK1/2 phosphorylation in response to thrombin was a prerequisite for the agonist-induced *de novo* synthesis of SM-MHC (18), one may speculate that after disruption of the PI 3-kinase signaling, PDGF-induced phenotypic modulation of VSM cells may shift toward differentiation. This hypothesis was addressed by transient transfection of a CAT-reporter construct expressed under the control of the −1346 nucleotide promoter region of the SM-MHC gene (pCAT-1346). VSM cells maintained in QM

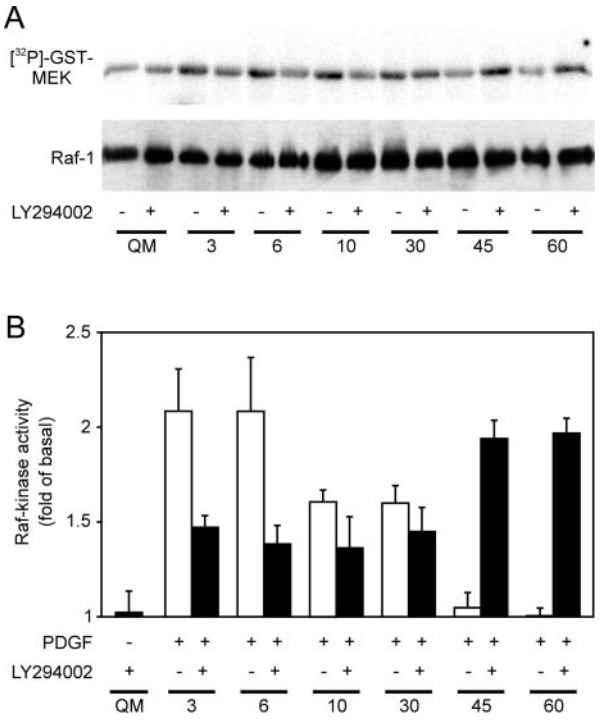


FIG. 5. **PI 3-kinase-dependent modulation of the PDGF-induced Raf activity.** VSM cells were preincubated with LY294002 (20 $\mu$M) or its solvent (0.05% $Me_2SO$) for 30 min and then stimulated with 10 ng/ml PDGF-BB for the indicated time (in min). The Raf kinase activity was assessed by immunoprecipitation of Raf-1 and coincubation of [$\gamma$-$^{32}$P]ATP and recombinant GST-MEK protein as a substrate. *A,* phosphorylated GST-MEK was separated by SDS-PAGE and assessed with a phosphorimaging system. The equal recovery of immunoprecipitated Raf was confirmed by applying anti-Raf-1 antibodies. Experiments were repeated three times with similar results, and a representative example is shown. *B,* statistical analysis of all experiments. The photon-induced luminescence of each sample was quantified and expressed as a fold induction of basal activity. The *bars* depict mean ± S.E. of three independent experiments for each time point.

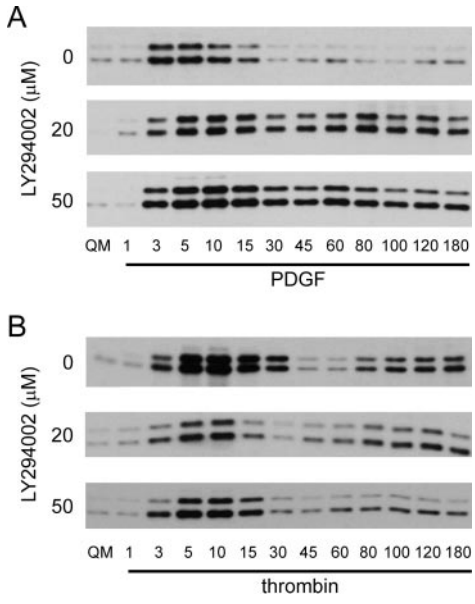


FIG. 6. **PI 3-kinase inhibition augments the PDGF-induced late-phase ERK phosphorylation.** *A,* whole cell lysates were prepared from VSM cells after treatment with different concentrations of LY294002 (added 30 min prior to agonist addition) and subsequent stimulation with PDGF (10 ng/ml for the indicated time in min). Phosphorylated ERK1/2 was detected as described in the legend to Fig. 2. *B,* comparative data for the thrombin (1 unit/ml)-induced temporal pattern of ERK1/2 phosphorylation with and without LY294002 pretreatment are given in the *lower panel*. Aliquots of the same lysates were probed with antibodies detecting total ERK1/2 demonstrating the equal loading (data not shown). Representative data of three to five independent experiments are shown.

showed an about 4-fold increased CAT activity as compared with controls transfected with a promoterless pCAT-basic vector. Under these conditions, the addition of PDGF for 36 h did not further increase the promoter activity (Fig. 7*A*). However, the pretreatment with LY294002 (50 $\mu$M) for 30 min prior to the addition of 10 ng/ml PDGF resulted in a more than 2-fold increase in CAT activity as compared with the absence of the PI 3-kinase inhibitor ($n = 6$). Similar results were obtained with wortmannin (100 nM). In the absence of PDGF, neither LY294002 nor wortmannin alone were sufficient to increase the promoter activity (data not shown).

To further substantiate that the effect of PI 3-kinase inhibition on the PDGF-induced SM-MHC promoter activity is mediated via Akt, genetically encoded modulators were applied. VSM cells were co-transfected with pCAT-1346 and different amounts of expression plasmids encoding dominant negative Akt (K179A mutant). In all co-transfection experiments, the total amount of transfected plasmid DNA was kept constant (1 $\mu$g/well) by addition of promoterless pCAT-basic. The CAT activity in PDGF-stimulated VSM cells was concentration dependently increased by coexpression of dominant negative Akt (Fig. 7*B*) corroborating the results with PI 3-kinase inhibitors. Thus, in PDGF-stimulated VSM cells, inhibition of PI 3-kinase/Akt extended ERK1/2 activity and up-regulated the SM-MHC promoter activity.

Conversely, constitutively active Akt should disrupt the differentiating signal of thrombin stimulation. The coexpression of Akt N-terminal fused to the myristoylation/palmitylation motif from the Lck tyrosine kinase (19) concentration dependently disrupted the thrombin-induced promoter CAT activation by more than 90% (Fig. 7*C*) similar to the action of dominant-negative Raf (18). These data demonstrate that sustained Raf/MEK/ERK signaling correlates with *in vitro* re-differentiation of VSM cells and is negatively controlled by the PI 3-kinase/Akt-pathway. Consistent with the hypothesis that the expression of contractile proteins may be controlled by a sustained ERK activation irrespective of the kind of the input signal, permanent activation of protein kinases C by phorbol 12-myristate 13-acetate (1–100 nM) induced a sustained ERK1/2 activation and increased the SM-MHC promoter activity in a concentration-dependent fashion up to the 2.3-fold at 10 nM (data not shown). Similarly, heterologous expression of constitutively active Raf (C-terminal fragment) increased the SM-MHC promoter activity by about 2-fold (data not shown). Hence, expression of contractile proteins in VSM cells is increased either by ligands inducing a sustained ERK activation or by suppression of PI 3-kinase/Akt that blocks sustained signaling through the Ras/Raf/MEK/ERK cascade at the level of Raf.

## DISCUSSION

In neonatal rat VSM cells, serum treatment induces proliferation but also increases the expression of contractile proteins. Although the serum components PDGF and thrombin activate similar signal transduction pathways, they have a divergent impact on mitogenesis and differentiation. Our results in VSM cells demonstrate that the balance of the PI 3-kinase/Akt and Ras/Raf/MEK/ERK activation corresponds to the proliferative and differentiating potential of the respective agonist. The PI 3-kinase-dependent activation of Akt results in an interaction with Raf that is accompanied by a phosphorylation at $Ser^{259}$, a decrease in Raf kinase activity, and subsequent reduced MEK and ERK1/2 phosphorylation. Thrombin

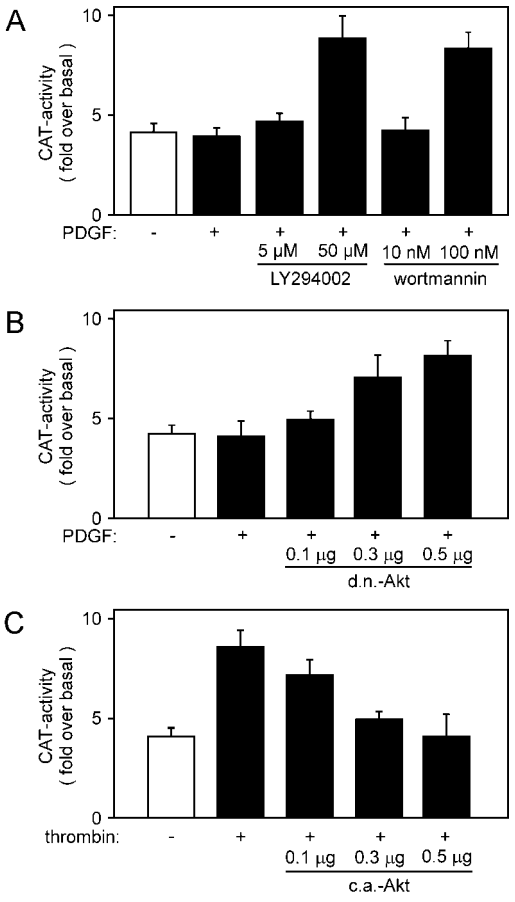


FIG. 7. **PI 3-kinase/Akt negatively regulate the PDGF-induced SM-MHC promoter activity.** *A,* VSM cells were transfected with a −1346 nucleotide SM-MHC promoter-CAT fusion construct (pCAT-1346) and then serum-starved for 36 h. Following pretreatment with the indicated concentrations of LY294002 or wortmannin for 30 min, cells were incubated in the presence of serum-free medium (*QM, white bar*) or QM supplemented with 10 ng/ml PDGF-BB (*black bars*). *B,* to test for the functional role of Akt in the PDGF-mediated SM-MHC promoter induction, VSM cells were co-transfected with 0.5 μg/well pCAT-1346 and the indicated amounts of dominant negative Akt expression constructs (*d.n. Akt*). The total amount of plasmid DNA was kept constant (1 μg/well) with promoterless pCAT-basic. *C,* VSM cells were co-transfected with pCAT-1346 and the indicated amounts of constitutively activate Akt (*c.a. Akt*) and then stimulated with 1 unit/ml thrombin. Cells were lysed 36 h after agonist application and analyzed for CAT activity. Depicted CAT activities were normalized for protein concentrations and compared with the CAT activity of cells transfected with a reporter gene construct lacking the SM-MHC promoter. *Bars* represent the mean ± S.E. of at least five independent transfection experiments.

induced a partial and temporary phosphorylation of Akt that was not sufficient to suppress the late-phase ERK activation. By contrast, the PDGF-induced strong and persistent phosphorylation of Akt inactivates Raf which terminates the coupling to MEK and ERK. Disruption of the PI 3-kinase/Akt signaling augmented the PDGF-induced Raf kinase activity resulting in a sustained ERK1/2 phosphorylation and subsequent activation of the SM-MHC promoter.

Both, receptor tyrosine kinases and G-protein-coupled receptors activate at least three common signaling modules: (i) PLC isoforms that increase the cytosolic $[Ca^{2+}]_i$ concentration and activate protein kinases C, (ii) the ERK class of MAP kinases, and (iii) the PI 3-kinase/Akt pathway. The balance of signal intensities, kinetics, and potential cross-regulations between these pathways may define the direction of phenotypic modulation in VSM cells. PDGF and thrombin induced a comparable extent and duration of $[Ca^{2+}]_i$ transients (18). Similarly, both

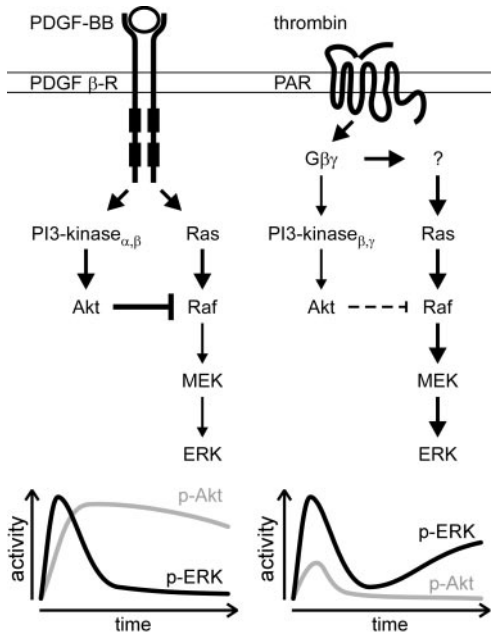


FIG. 8. **Schematic diagram of the PDGF- and thrombin-mediated regulation of ERKs.** PI 3-kinase-dependent sustained Akt activation following ligand binding of PDGF β-receptors inhibits Raf kinase activity and suppresses MEK and subsequent late phase ERK activation. The short-lived Akt activity following stimulation of a protease-activated receptor fails to inhibit Raf kinase activity. The Gβγ-induced activation of the Ras/Raf/MEK cascade results in a second-phase ERK phosphorylation required for the enhanced expression of contractile proteins. *PDGF β-R,* PGDF β-receptor; *PAR,* protease-activated receptor; *PI 3-kinases*$_{\alpha,\beta,\gamma}$, α-, β-, and γ-subtypes of phosphatidylinositol-3-kinase; *p-Akt,* activated (S-473-phosphorylated) Akt; *p-ERK,* phosphorylated p42/p44-forms of extracellular signal related kinase.

receptors initiated a rapid and almost complete phosphorylation of ERK1/2 as deduced by mobility shifts of protein bands when whole cell lysates were analyzed with anti-ERK1/2 antibodies. At later time points, however, a second-phase ERK1/2 phosphorylation was only detectable when cells were stimulated with thrombin. More strikingly, Akt was only weakly and transiently phosphorylated by thrombin treatment, whereas PDGF induced a robust and long-lived Akt phosphorylation. This differential kinetic pattern of Akt activation may be due to the different equipment of the cells with various PI 3-kinase isoforms. There is a large body of evidence that G-protein-coupled receptors via Gβγ subunits are capable of activating the β- and γ-isoforms of class I PI 3-kinases (20–23). Receptor tyrosine kinases couple to PI 3-kinase α (24, 25) and, in synergism with Gβγ, activate PI 3-kinase β (26).

The weak and short-lived Akt phosphorylation in response to thrombin treatment may rely on a reduced availability of Gβγ subunits. RGS3 has been demonstrated to limit Gβγ-dependent activation of both Akt and ERK1/2 by virtue of its GAP activity for $G\alpha_i$ subunits and subsequent reassociation of the GDP-bound $G\alpha_i$ and Gβγ (27). Alternatively, Akt may be more rapidly dephosphorylated by ceramide-sensitive (28) or other protein phosphatases. This open question is currently addressed by monitoring the translocation of phosphoinositide 3,4,5-$P_3$-sensitive pleckstrin homology domains. The GFP-fused pleckstrin homology domains of Akt and GRP1 (29) may be valuable tools for this attempt.

Fig. 8 depicts a model of the PDGF- and thrombin-induced signaling to the PI 3-kinase/Akt and Ras/Raf/MEK/ERK cascades that includes a negative cross-regulation of Raf by activated Akt. The coupling to PI 3-kinases and subsequent Akt phosphorylation upon PDGF stimulation elicits a strong and persistent signal that remained detectable for several hours.

Since an interference of the Ras/Raf/MEK/ERK and PI 3-kinase/Akt cascades has been demonstrated and previously attributed to direct interaction and inhibitory phosphorylation of Raf by Akt (13, 14), we studied the role of this cross-regulation for the PDGF-mediated signaling in VSM cells. Indeed, the strong phosphorylation of Akt coincided with an association with c-Raf and its phosphorylation at $Ser^{259}$ (Figs. 3 and 4), a critical position for the intrinsic Raf kinase activity (30). Furthermore, our data demonstrate the PI 3-kinase dependence of Akt and Raf activities and its inhibitory consequence on MEK and ERK1/2 phosphorylation. Consistent with the idea that a prolonged ERK activity is necessary and sufficient to up-regulate the expression of contractile proteins, the shift of the balance toward sustained Raf/MEK/ERK signaling adds a differentiating potential to a well accepted mitogen in VSM cells. In agreement with the hypothesis that the mitogenic signal may rely on the inhibitory cross-regulation of the Ras/Raf/MEK/ERK cascade by Akt, overexpression of Akt has been shown to override the NGF-induced growth arrest and to inhibit the differentiation of PC12 cells (31). Moreover, the observed PI 3-kinase/Akt-mediated termination of late-phase ERK1/2 phosphorylation may serve as a mechanistical explanation for the temporal pattern of short-lived ERK1/2 phosphorylation followed by a more delayed PI 3-kinase activity that is critical for the PDGF-induced progression of the cell cycle from $G_1$ to S (32).

Besides the inactivating phosphorylation through Akt, Raf isoforms underlie various further modulating input signals. An alternative termination signal is given by the docking of p120-Ras-GAP to the activated PDGF $\beta$-receptor. Although becoming phosphorylated at the ligand-bound PDGF $\beta$-receptor, it is not certain whether the phosphorylation of p120-Ras-GAP affects its GTPase accelerating activity at the downstream effector Ras. In p120-Ras-GAP knock-out mice, however, PDGF-BB treatment has been demonstrated to enhance and prolong the phosphorylation of ERK2 (33). In transfection experiments applying mutated PDGF $\beta$-receptors, the single readdition of the p120-Ras-GAP docking site $Tyr^{771}$ largely suppresses the PDGF-induced mitogenis by silencing PLC-$\gamma$ (34). The additional finding that phosphorylated p120-Ras-GAP disrupts the ability of Src to promote the phosphorylation of PLC-$\gamma$ (35) offers an unexpected and Ras-independent role of this signaling molecule in the supramolecular signaling complex of the receptor tyrosine kinase. The possible role of p120-Ras-GAP for the PDGF-induced mitogenic signaling in VSM cells, however, remains to be clarified.

Besides c-Raf, other Raf isoforms may transmit the late-phase ERK phosphorylation. In PC12 cells, the NGF-induced early ERK phosphorylation via Ras and c-Raf is followed by a second wave ERK phosphorylation via activated Rap1 which forms a stable complex with B-Raf (36). This alternative pathway to promote a long-lived activation of ERKs, however, is equally sensitive to negative regulation by Akt (37). Indeed, overexpressed Akt overrides the NGF-induced growth arrest and blocks the neurite outgrowth in PC12 cells (31). The protein kinase A-mediated inhibitory phosphorylations of Raf (38–40) or the Ras-dependent activation of Raf by protein kinases C (41, 42) assemble to a complex picture of signal integration at the level of Raf.

The thrombin-induced re-differentiation of VSM cells correlates with activation of ERK and can be mimicked by the heterologous expression of an activated Raf mutant. The degree of excess activation of the Raf/MEK/ERK kinase cascade is critical to cell survival since transfection of highly active Raf mutants led to detachment of cells indicative of a toxic effect (data not shown). The S259A replacement of Raf used in this study interferes with binding of 14-3-3 (43) and may keep Raf in a open conformation. Previous studies have been shown that S259A-Raf is about 2.5-fold more active than the wild-type molecule (30), an effect that is sufficient to increase the expression of SM-MHC in VSM cells (18). Similarly, activation of endogenous Raf by stimulating receptor tyrosine kinases with IGF, epidermal growth factor, or NGF revealed an about 2–3-fold activation of the Raf kinase activity (44, 45). In PDGF-stimulated VSM cells, PI 3-kinase inhibition increased the late-phase Raf activity by about 2-fold, an effect that was sufficient to generate a prolonged ERK1/2 phosphorylation. Moreover, the appearance of a more sustained activation of ERKs closely correlated with the gain of SM-MHC promoter activation in response to PDGF stimulation. It is tempting to speculate that, by reducing the PI 3-kinase/Akt-mediated negative input into the Raf kinase, the balance of PDGF-induced signaling pathways now resembles that seen after thrombin stimulation. The outcome of the cells may therefore be shifted toward differentiation. These findings correlate with each other in terms of the activation of Raf and ERK1/2, the degree of the induction of the biological response, and the behavior of the activated Raf mutants.

Hayashi *et al.* (46) found that the induction of PI 3-kinase activity through IGF or insulin is essential to maintain the differentiated phenotype of embryonic gizzard smooth muscle cells when cultured on laminin (46). In these cells IGF-I failed to activate ERK1/2, JNK, or p38 MAP kinase. In vascular smooth muscle cells, however, several reports and our findings demonstrate that IGF-I stimulates MAP kinases and promotes proliferation and migration of vascular smooth muscle cells (47, 48). In contrast to our results with VSM cells, the maintenance of the differentiated state of the visceral smooth muscle was attributed to the IGF-I-induced PI 3-kinase activity (46). Although their concept was extended to freshly isolated aortic smooth muscle cells (49), the lack of a biochemical characterization of the processes precludes the comparison of these data with our results. Consistent with the role of Akt to override growth arrest in PC12 cells, PI 3-kinase signaling results in growth and oncogenic transformation in a variety of cell types (50, 51). The puzzling finding that PI 3-kinase signaling exhibits both differentiation or proliferative and even oncogenic potential may rely on differential effects of PI 3-kinase isotypes, cell-specific expression of their effector molecules, and downstream integration with other coincident signaling mechanisms. Besides its lipid kinase activity, class I PI 3-kinases directly bind to Ras (52), but only PI 3-kinase $\alpha$ has been shown to be directly stimulated by Ras-GTP (53). On the other hand, PI 3-kinase $\gamma$ has been shown to decrease the GTPase activity of Ras (54). Thus, the sequential activation of these signaling molecules is still under debate. The counteracting phosphatidylinositol-3-phosphatase PTEN is widely accepted to mediate growth arrest (55) and was initially characterized as a tumor suppressor molecule (56). In agreement with this concept, our data point to a role of PI 3-kinase/Akt to promote the PDGF-mediated growth and de-differentiation of VSM cells. The sustained ERK1/2 phosphorylation and/or the absence of PI 3-kinase activation may serve the appropriate signal to induce growth arrest and differentiation. Pharmacological or genetic modulation of the balance between signaling cascades may therefore serve as a target to propagate the differentiated state of vascular smooth muscle.

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REFERENCES

1. Jawien, A., Bowen-Pope, D. F., Lindner, V., Schwartz, S. M., and Clowes, A. W. (1992) *J. Clin. Invest.* **89,** 507–511
2. Jackson, C. L., Raines, E. W., Ross, R., and Reidy, M. A. (1993) *Arterioscler. Thromb.* **13,** 1218–1226
3. Arnqvist, H. J., Bornfeldt, K. E., Chen, Y., and Lindstrom, T. (1995) *Metabolism* **44,** 58–66
4. Heldin, C. H., Ostman, A., and Ronnstrand, L. (1998) *Biochim. Biophys. Acta* **1378,** 79–113
5. Marshall, C. J. (1995) *Cell* **80,** 179–185
6. Pawson, T. (1995) *Nature* **373,** 573–580
7. Heldin, C. H. (1995) *Cell* **80,** 213–223
8. Kandel, E. S., and Hay, N. (1999) *Exp. Cell Res.* **253,** 210–229
9. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551
10. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91,** 231–241
11. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., and Blenis, J. (1999) *Cell* **96,** 857–868
12. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282,** 1318–1321
13. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D., and Glass, D. J. (1999) *Science* **286,** 1738–1741
14. Zimmermann, S., and Moelling, K. (1999) *Science* **286,** 1741–1744
15. Ives, H. E., Schultz, G. S., Galardy, R. E., and Jamieson, J. D. (1978) *J. Exp. Med.* **148,** 1400–1413
16. Madsen, C. S., Hershey, J. C., Hautmann, M. B., White, S. L., and Owens, G. K. (1997) *J. Biol. Chem.* **272,** 6332–6340
17. Reusch, H. P., Wagdy, H., Reusch, R., Wilson, E., and Ives, H. E. (1996) *Circ. Res.* **79,** 1046–1053
18. Reusch, H. P., Schaefer, M., Plum, C., Schultz, G., and Paul, M. (2001) *J. Biol. Chem.* **276,** 19540–19547
19. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 31515–31524
20. Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nürnberg, B., Gierschik, P., Seedorf, K., Hsuan, J. J., Waterfield, M. D., and Wetzker, R. (1995) *Science* **269,** 690–693
21. Tang, X., and Downes, C. P. (1997) *J. Biol. Chem.* **272,** 14193–14199
22. Leopoldt, D., Hanck, T., Exner, T., Maier, U., Wetzker, R., and Nürnberg, B. (1998) *J. Biol. Chem.* **273,** 7024–7029
23. Murga, C., Fukuhara, S., and Gutkind, J. S. (2000) *J. Biol. Chem.* **275,** 12069–12073
24. Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A., and Williams, L. T. (1991) *Cell* **65,** 75–82
25. McGlade, C. J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A. D., Panayotou, G., End, P., Bernstein, A., and Kazlauskas, A. (1992) *Mol. Cell. Biol.* **12,** 991–997
26. Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Ui, M., Hazeki, O., and Katada, T. (1997) *J. Biol. Chem.* **272,** 24252–24256
27. Shi, C.-S., Lee, S. B., Sinnarajah, S., Dessauer, C. W., Rhee, S. G., and Kehrl, J. H. (2001) *J. Biol. Chem.* **276,** 24293–24300
28. Schubert, K. M., Scheid, M. P., and Duronio, V. (2000) *J. Biol. Chem.* **275,** 13330–13335
29. Gray, A., Van Der Kaay, J., and Downes, C. P. (1999) *Biochem. J.* **344,** 929–936
30. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) *J. Biol. Chem.* **268,** 17309–17316
31. Bang, O. S., Park, E. K., Yang, S. I., Lee, S. R., Franke, T. F., and Kang, S. S. (2001) *J. Cell Sci.* **114,** 81–88
32. Jones, S. M., Klinghoffer, R., Prestwich, G. D., Toker, A., and Kazlauskas, A. (1999) *Curr. Biol.* **9,** 512–521
33. van der Geer, P., Henkemeyer, M., Jacks, T., and Pawson, T. (1997) *Mol. Cell. Biol.* **17,** 1840–1847
34. Valius, M., Secrist, J. P., and Kazlauskas, A. (1995) *Mol. Cell. Biol.* **15,** 3058–3071
35. Schlesinger, T. K., Demali, K. A., Johnson, G. L., and Kazlauskas, A. (1999) *Biochem. J.* **344,** 519–526
36. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. (1998) *Nature* **392,** 622–626
37. Guan, K. L., Figueroa, C., Brtva, T. R., Zhu, T., Taylor, J., Barber, T. D., and Vojtek, A. B. (2000) *J. Biol. Chem.* **275,** 27354–27359
38. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993) *Science* **262,** 1065–1069
39. Schramm, K., Niehof, M., Radziwill, G., Rommel, C., and Moelling, K. (1994) *Biochem. Biophys. Res. Commun.* **201,** 740–747
40. Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A., and Kolch, W. (1996) *Mol. Cell. Biol.* **16,** 5409–5418
41. Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F., and Marshall, C. J. (1998) *Science* **280,** 109–112
42. Schönwasser, D. C., Marais, R. M., Marshall, C. J., and Parker, P. J. (1998) *Mol. Cell. Biol.* **18,** 790–798
43. Rommel, C., Radziwill, G., Lovric, J., Noeldeke, J., Heinicke, T., Jones, D., Aitken, A., and Moelling, K. (1996) *Oncogene* **12,** 609–619
44. Soderholm, H., Olsson, A., Lavenius, E., Ronnstrand, L., and Nanberg, E. (2001) *Cell. Signal.* **13,** 95–104
45. Kim, J., Yoon, L. Y., Choi, S. L., Kang, I., Kim, S. S., Kim, Y. S., Choi, Y. H., and Ha, J. (2001) *J. Biol. Chem.* **276,** 19102–19110
46. Hayashi, K., Saga, H., Chimori, Y., Kimura, K., Yamanaka, Y., and Sobue, K. (1998) *J. Biol. Chem.* **273,** 28860–28867
47. Delafontaine, P., Lou, H., and Alexander, R. W. (1991) *Hypertension* **18,** 742–747
48. Bornfeldt, K. E., Raines, E. W., Nakano, T., Graves, L. M., Krebs, E. G., and Ross, R. (1994) *J. Clin. Invest.* **93,** 1266–1274
49. Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H., and Sobue, K. (1999) *J. Cell Biol.* **145,** 727–740
50. Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B., and Gray, J. W. (1999) *Nat. Genet.* **21,** 99–102
51. Aoki, M., Batista, O., Bellacosa, A., Tsichlis, P., and Vogt, P. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14950–14955
52. Pacold, M. E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C. T., Walker, E. H., Hawkins, P. T., Stephens, L., Eccleston, J. F., and Williams R. L. (2000) *Cell* **103,** 931–943
53. Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) *Nature* **370,** 527–532
54. Rubio, I., Wittig, U., Meyer, C., Heinze, R., Kadereit, D., Waldmann, H., Downward, J., and Wetzker, R. (1999) *Eur. J. Biochem.* **266,** 70–82
55. Li, D. M., and Sun, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15406–15411
56. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. (1997) *Science* **275,** 1943–1947