Systematic Evaluation of Anti-apoptotic Growth Factor Signaling in Vascular Smooth Muscle Cells

ONLY PHOSPHATIDYLINOSITOL 3'-KINASE IS IMPORTANT*

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Peptide growth factors contribute to the pathogenesis of cardiovascular diseases by inducing a variety of cellular responses including anti-apoptotic effects. Several of the signaling molecules that are activated by growth factor receptors such as Src family kinases (Src), phosphatidylinositol 3'-kinase (PI3K), phospholipase Cγ (PLCγ), Ras, and SHP-2 were shown to mediate survival signals. We systematically investigated the relative contribution of each signaling molecule for growth factor-dependent cell survival in vascular smooth muscle cells (VSMC). Our approach was the use of mutated platelet-derived growth factor (PDGF) β-receptors (βPDGFR) in which the tyrosine residues required for binding of each signaling molecule were individually mutated to phenylalanine. To bypass endogenous PDGFR in VSMC we used chimeric receptors (ChiRs), containing the extracellular domain of the macrophage colony-stimulating factor (M-CSF) receptor and the cytoplasmic domain of the wild type (WT) or mutated βPDGFR. Selective activation of the ChiR-WT with M-CSF significantly reduced apoptosis to the same extent as PDGF-BB in non-transfected cells. Deletion of the binding site for PI3K, but not for Src, RasGAP, SHP-2, or PLCγ, completely abolished the anti-apoptotic effect. Consistently, a ChiR mutant that only binds PI3K was fully able to mediate cell survival as efficiently as the ChiR-WT. Furthermore, the PDGF-dependent anti-apoptotic effect in non-transfected cells was completely abolished by the PI3K inhibitor wortmannin, whereas inhibitors of Src, PLCγ, ERK, or p38 MAP kinase had no effect. The exploration of downstream signaling events revealed that PDGF-BB activates the anti-apoptotic Akt signaling pathway in a PI3K-dependent manner. Moreover, Akt phosphorylates and thus inactivates the pro-apoptotic proteins BAD and Forkhead transcription factors (FKHR, FKHL1). We conclude that growth factor-dependent cell survival in VSMC is mediated only by activation of the PI3K/Akt pathway, whereas all other receptor-associated signaling molecules do not play a significant role.

The pathogenesis of vascular diseases involves an abnormal accumulation of lipids, inflammatory cells, and vascular smooth muscle cells (VSMC) within the intimal layer of the vessel wall. Recent studies have demonstrated that in addition to cell proliferation and migration, dysregulated apoptosis plays a prominent role in the pathogenesis and progression of atherosclerosis (1). Peptide growth factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor-I are potent inhibitors of cell death. These growth factors exert their biological responses via interaction of transmembrane receptor tyrosine kinases. Upon ligand binding, receptor tyrosine kinases autophosphorylate on tyrosine residues and subsequently recruit and activate SH2 domain-containing cytoplasmic signaling molecules such as Src family kinases (Src), the GTPase activating protein of Ras (RasGAP), the phosphotyrosine phosphatase SHP-2, phosphatidylinositol 3' kinase (PI3K), and phospholipase Cγ (PLCγ) (2).

Recent studies indicated that all of these signaling molecules are implicated in survival signaling and therefore may contribute to growth factor-mediated cell survival. In PDGF-stimulated cells, Src family kinases are required for activation of STAT (signal transducer and activator of transcription) 3 (3, 4), which protects fibroblasts from apoptosis and antagonizes the pro-apoptotic effects of activated STAT1 (5). In addition, Src was shown to mediate anti-apoptotic effects by activating PI3K (6). PI3K mediates anti-apoptotic signals of several growth factors via activation of its downstream target Akt (7). The serine/threonine kinase Akt (8, 9) phosphorylates and thus inactivates several pro-apoptotic proteins such as BAD (10), Forkhead transcription factors or caspase-9 (11, 12). Further downstream mediators of this pathway include NFXβ and glycogen synthase kinase-3β (GSK3β) (9, 13). Recently, SHP-2 was shown to be essential for insulin-like growth factor-I- or PDGF-mediated cell survival, as it suppresses caspase 3-dependent apoptosis in fibroblasts (14). Another well-characterized survival pathway is the Ras/Raf/extracellular-regulated kinase (ERK) cascade, which leads to the phosphorylation of BAD (15, 16). Finally, PLCγ is required for insulin-like growth factor-I-mediated protection against apoptosis (17), as it medi-
ates cell survival via protein kinase C-dependent phosphorylation of Bcl-2 proteins such as Bcl-2 and BAD (18–20).

Each of the above studies investigated only one of the growth factor-activated signal relay enzymes, and most studies used either pharmacological inhibitors, which may lack specificity, or a dominant-negative approach, which may influence cell survival by overexpressed protein levels. Additionally, inhibitors as well as overexpressed dominant-negative proteins suppress the basal activity of the inhibited signaling molecule in unstimulated cells, limiting the significance about their role for ligand-induced cellular responses. Hence, the relative roles of Src, PLCγ, RasGAP, PI3K, and SHP-2 for growth factor-mediated cell survival are not clear from these studies. Therefore, we sought to systematically evaluate the relative contribution of each of the above signaling molecules for growth factor-mediated cell survival in one system. We chose the PDGF β-receptor (βPDGFR), because this receptor tyrosine kinase, which is highly relevant for atherogenesis and tumorigenesis, binds each of the receptor-associated signal relay enzymes at one specific binding site. Our approach was to characterize βPDGFR mutants in which the binding sites for one or more of the receptor-associated signaling enzymes were individually deleted by selective tyrosine to phenylalanine substitutions. Importantly, we studied PDGFR signaling in VSMC, which are critically involved in atherogenesis, and in which PDGFR signaling is physiologically relevant, as these cells express endogenous PDGFR and thus respond to PDGF. However, to selectively stimulate PDGFR mutants in PDGFR-expressing cells, it is necessary to bypass endogenous βPDGFR. Therefore, we used chimeric M-CSF receptor (CSF1R)/βPDGFR mutants (ChiR) containing the extracellular domain of the CSF1R, which is not expressed in naive VSMC, and the cytoplasmic domain of the βPDGFR. By these chimeras, βPDGFR signaling is selectively stimulated with M-CSF. The use of this highly efficient system allowed us to selectively activate individual signaling molecules that are recruited to the ligand-stimulated βPDGFR and to compare the ability of the various ChiR mutants to protect VSMC against apoptosis.

MATERIALS AND METHODS

Cell Culture—VSMC were isolated from rat thoracic aorta (Wistar Kyoto; 6–10 weeks old; Charles River Wega GmbH, Sulzfeld, Germany) by enzymatic dispersion as described (21). Cells were grown in a 5% CO2 atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids (100×), and 10% fetal calf serum.

Generation and Expression of ChiR Mutants—The ChiR cDNA was constructed from DNA encoding amino acids 1–513 of the human CSF1R and amino acids 528–1106 of the human PDGFR joined at an EcoRI site (22). Mutations in the βPDGFR sequence are as described (23–26) and were obtained by site-directed mutagenesis and subcloning. Constructs were assembled in the pLXSN retroviral expression vector (27), sequence-verified, and transfected into the 293T packaging cell line as described (28). Forty-eight hours after transfection, retroviral supernatants were added to VSMC. Positive infectants were selected with 0.5 mg/ml G418.

Immunoprecipitation and Western Blot Analysis—Quiescent VSMC were left resting or stimulated with 50 ng/ml PDGF-BB or M-CSF for times indicated in the presence or absence of inhibitors as indicated. Cells were washed twice with HS (20 μM Hepes, pH 7.4, 150 mM NaCl) and then lysed in EB (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% bovine serum albumin, 20 μg/ml aprotinin, 2 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged (20 min, 12,000 × g), and the supernatants were subjected to immunoblot analysis or immunoprecipitation. The βPDGFR and the ChiRs were precipitated with a polyclonal βPDGFR antibody (97A) recognizing the kinase insert (amino acids 698–797) of the human βPDGFR. βPDGFR immunoprecipitations representing ~3 × 106 cells were resolved on a 7.5% SDS-polyacrylamide gel, and the proteins were transferred to Immobilon and subjected to Western blot analysis. To monitor the association of signaling molecules with the activated βPDGFR, receptor immunoprecipitations from resting or M-CSF-stimulated cells were subjected to Western blot analysis using antibodies against the extracellular domain of the human CSF1R (anti-CSF1R), PLCγ, RasGAP, p85, and SHP-2. To monitor the association between Src and the ChiRs, Src was immunoprecipitated from resting or M-CSF-stimulated cells using the Src-2 antibody, and immunoprecipitates representing ~3 × 106 cells were subjected to anti-CSF1R or anti-Src Western blot analysis. BAD was precipitated with a monoclonal BAD antibody. Immune complexes were bound to Western blot analysis using a polyclonal BAD antibody or a phosphospecific (Ser-136) BAD antibody. To monitor the expression levels of ChiR mutants in VSMC and the phosphorylation state of downstream signaling subunits, whole cell lysates representing ~3 × 106 cells were resolved on a 10% SDS-polyacrylamide gel and probed with anti-CSF1R, and antibodies were raised against RasGAP (lyase control), BAD and phosphorylated Akt, GSK3β, BAD, FKHR, and FKHR. The results presented are representative of at least three independent experiments.

Induction and Detection of Apoptosis—Apoptosis was induced by either UV irradiation (254 nm, 70 mJ/cm2) or H2O2 (100 μM). A cell death detection ELISA (Roche Diagnostics) was used to detect cell death by the detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Cells (5,000 cells/well) were plated into 96-well plates in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and starved for 24 h in Dulbecco’s modified Eagle’s medium. To assess the anti-apoptotic effect of PDGF-BB (50 ng/ml) or M-CSF (50 ng/ml), the cells were UV light-irradiated and subsequently cultured for 8 h in the absence or presence of ligand. Alternatively, the cells were incubated for 12 h in Dulbecco’s modified Eagle’s medium containing H2O2 in the presence or absence of PDGF-BB or M-CSF. The cell lysates were collected, and apoptosis was measured using the cell death detection ELISA.

Materials—PDGF-BB and M-CSF were purchased from Promega (Madison, WI). The monoclonal antibody raised against the extracellular domain of the human CSF1R was from Upstate Biotechnology. The anti-phosphotyrosine antibodies were purchased from Oncogene Science Inc., was used. The polyclonal phospho-Akt (Ser-473), phospho-FKHR (Thr-24)/FKHRL1 (Thr-32), phospho-Bad (Ser-136), Akt, and BAD antibodies were from Cell Signaling Technologies. The monoclonal BAD antibody was from BD Biosciences.

Statistical Analysis—All data are expressed as means ± S.D. Statistical analysis was evaluated by non-parametric analysis. p < 0.05 was considered significant.

RESULTS

Characterization of Chimeric CSF1R/βPDGFR Mutants—To systematically evaluate the role of each of the βPDGFR signaling molecules (Src, PI3K, RasGAP, SHP-2, and PLCγ) for growth factor-mediated cell survival in a single cell type, we created a series of ChiR in which the tyrosine residues required for the association of each of the above signaling enzymes were individually mutated to phenylalanine (subtraction mutants) (27, 28). A second set of ChiR (addition mutants) was based on the F5 receptor, in which the 5 tyrosine phosphorylation sites responsible for binding of PI3K, RasGAP, SHP-2, and PLCγ were mutated. This panel of receptors included mutants that have the tyrosine residue(s) required for binding of one of the associated proteins restored (Fig. 1B). In this panel, the Src-binding sites were left intact, as their mutation in the juxtamembrane region was shown to attenuate the ligand-induced tyrosine phosphorylation of the full-length βPDGFR (25). All ChiR were stably expressed in VSMC. Additionally, an “F6” receptor was expressed, which carries an additional mu-
The cytoplasmic domain is shown as a schematic in which the tyrosine phosphorylation sites are represented as $P$, and Tyr to Phe substitutions are indicated as black squares. Signaling enzymes predicted to stably associate with the receptor mutants are indicated by geometric shapes and are identified at the top of the schemes. The nomenclature of the “subtraction panel” (A) and “add-back panel” (B) of the ChiR is indicated to the right of each receptor representation. In the subtraction panel, the names indicate which of the tyrosine residues have been replaced with phenylalanine, and in the add-back panel the name of each mutant denotes which of the mutations in the F5 construct has been repaired. Tyr-579 and -581 are located in the juxtamembrane domain (JM) of the receptor and are required for Src binding; Tyr-741, -752, and -771 are in the kinase insert (KI) of the receptor and are responsible for PI3K and RasGAP binding, respectively; Tyr-1009 and -1021 are located in the C-terminal tail region of the receptor and are involved in the binding of SHP-2 and PLCγ, respectively. The F6 construct contains an additional mutation at the Grb2-binding site Tyr-716.

**Fig. 1.** Schematic diagram illustrating the series of ChiR used in this study. The cytoplasmic domain is shown as a schematic in which the tyrosine phosphorylation sites are represented as $P$, and Tyr to Phe substitutions are indicated as black squares. Signaling enzymes predicted to stably associate with the receptor mutants are indicated by geometric shapes and are identified at the top of the schemes. The nomenclature of the “subtraction panel” (A) and “add-back panel” (B) of the ChiR is indicated to the right of each receptor representation. In the subtraction panel, the names indicate which of the tyrosine residues have been replaced with phenylalanine, and in the add-back panel the name of each mutant denotes which of the mutations in the F5 construct has been repaired. Tyr-579 and -581 are located in the juxtamembrane domain (JM) of the receptor and are required for Src binding; Tyr-741, -752, and -771 are in the kinase insert (KI) of the receptor and are responsible for PI3K and RasGAP binding, respectively; Tyr-1009 and -1021 are located in the C-terminal tail region of the receptor and are involved in the binding of SHP-2 and PLCγ, respectively. The F6 construct contains an additional mutation at the Grb2-binding site Tyr-716.

**Fig. 2.** Western blot analysis of receptor autophosphorylation and binding of signaling molecules. To investigate the ligand-induced autophosphorylation of the receptor and M-CSF-dependent binding of signaling molecules, ChiRs were immunoprecipitated from resting or M-CSF-stimulated cells, and the samples were subjected to Western blot analysis using antibodies against the extracellular domain of the CSF1R and against each of the signal relay enzymes. In addition, the receptor blot was stripped and reprobed with an anti-phosphotyrosine antibody. As shown in Fig. 2, A and D, all ChiR were expressed at equal levels.

**Fig. 2** demonstrates the binding characteristics of the add-back panel of ChiRs. Restoration of the PLCγ, RasGAP, and PI3K-binding sites at tyrosines 1021, 771, and 740/51 rescued the M-CSF-dependent association with PLCγ, RasGAP, and...
Mutants were left resting (phosphorylation and association with signaling molecules. Quiescent VSMC expressing either the ChiR-WT or the various phosphorylation site recognizing the H9252 demonstrate that the ChiR mutant system works sufficiently in ated signaling molecules upon M-CSF stimulation. These data Resting VSMC expressing the various constructs were lysed and subjected to Western blot analysis using antibodies against the extracellular domain of the M-CSF receptor (CSF1R) (top panel) or RasGAP as a lysate control (bottom panel). B, M-CSF-dependent tyrosine phosphorylation and association with signaling molecules. Quiescent VSMC expressing either the ChiR-WT or the various phosphorylation site mutants were left resting (-) or stimulated with M-CSF (+) for 5 min. Cell lysates were subjected to immunoprecipitation (IP) with an antiserum recognizing the βPDGFR. Immunoprecipitates were analyzed by Western blotting. Immunoblotting with anti-CSF1R revealed that there were similar amounts of ChiR present in all of the samples. The receptor blot was stripped and reprobed with a mixture of anti-phosphotyrosine antisera. Western blot analysis of immunoprecipitates was also performed using antisera against p85, RasGAP, PLCγ, and SHP-2 to detect co-immunoprecipitation of these signaling molecules with the activated ChiR. C, M-CSF-dependent association of ChiR-WT, F79/81, and R634 with Src. The R634 receptor has lysine at position 634 replaced with arginine and has no detectable in vitro kinase activity and was used as a negative control. Cells were stimulated and lysed and the lysates were immunoprecipitated with an antiserum that recognizes multiple Src family members. Immunoprecipitates were then immunoblotted with anti-CSF1R to detect coimmunoprecipitation of the ChiR mutants (upper panel) or with anti-Src to determine the amount of Src in each of the samples (lower panel). D and E, expression levels, tyrosine phosphorylation, and binding characteristics of the add-back panel of ChiR mutants. The analysis was done as described under A and B, D, expression levels. E, M-CSF-dep- dent tyrosine phosphorylation and association with signaling molecules. The results presented are representative of at least three independent experiments.

p85, respectively, whereas none of the other signal relay enzymes associated with these receptors. All add-back mutants associated with trace amounts of p85. Surprisingly, SHP-2 bound to the activated F5 receptor and to all add-back mutants, with the exception of the F6 and Y40/51 receptors. This suggests that SHP-2 is able to associate with at least one additional binding site besides tyrosine 1009 and, furthermore, that either binding or activation of PI3K prevents SHP-2 binding to this or these additional site(s). Rönstrand et al. (30) have reported that SHP-2 is also capable to bind to phosphorylated tyrosine 763, which is relatively close to the PI3K-binding site. This suggests that PI3K binding may sterically hinder the association of SHP-2 with phosphorylated tyrosine 763. R634, which served as a negative control in the biological assays, was not autophosphorylated, and failed to recruit βPDGFR-associated signaling molecules upon M-CSF stimulation. These data demonstrate that the ChiR mutant system works sufficiently in VSMC and enabled us to reliably investigate anti-apoptotic βPDGFR signaling.

M-CSF Mimics PDGF-dependent Cell Survival in ChiR-WT-expressing VSMC—To confirm that the activated ChiR-WT mimics PDGF-BB-dependent survival responses we compared the apoptosis rate of PDGF-stimulated non-transfected VSMC with that of M-CSF-treated ChiR-WT-expressing VSMC upon UV irradiation or H2O2 treatment. Irradiation and H2O2 treatment led to a 2.13 ± 0.54 and 5.31 ± 1.12-fold increase in cell death, respectively (Fig. 3A). The supplementation of culture medium with 50 ng/ml PDGF-BB significantly decreased irradiation- and H2O2-induced cell death by 48 ± 14 and 41 ± 21%, respectively (both p < 0.05). Consistently, irradiation or treatment of ChiR-WT-expressing VSMC with H2O2 caused a 1.59 ± 0.17 and 2.51 ± 0.27-fold increase in apoptotic cell death, respectively, compared with non-treated cells. Co-incubation with M-CSF (50 ng/ml) significantly reduced the rate of irradiation- or H2O2-induced apoptosis by 42 ± 12 and 40 ± 11%, respectively (both p < 0.05), whereas it had no effect on cell survival in non-transfected VSMC (not shown). Hence, stimulation of βPDGFR signaling by M-CSF in ChiR-WT-expressing VSMC was sufficient to mediate PDGF-induced responses to the same extent as PDGFB-B in non-transfected cells.

Systematic Evaluation of βPDGFR-mediated Anti-apoptotic Signaling in VSMC—To investigate the role of each of the signaling molecules for βPDGFR-induced protection from apoptosis, we compared this response in VSMC expressing either ChiR-WT or the various mutant receptors. We induced apoptosis by UV irradiation of quiescent cells. An analysis of the subtraction panel revealed that deletion of the binding site for PI3K (F40/51), but not for Src, RasGAP, SHP-2, or PLCγ, completely suppressed the anti-apoptotic effect (Fig. 3B). Consistently, binding and activation of only PI3K without activa-
tion of RasGAP, SHP-2, and PLCγ via the Y40/51 receptor was sufficient to mediate the complete M-CSF-induced anti-apoptotic effect. In contrast, all other add-back mutants were unable to mediate a significant anti-apoptotic effect (Fig. 3C).

To exclude the possibility that our findings were limited to one specific inducer of apoptosis, we repeated our experiments when apoptosis was induced by H2O2. Similar to the above studies, a lack of PI3K binding (F40/51) completely abolished the anti-apoptotic effect mediated by the ChiR-WT, whereas restoration of the PI3K-binding site to the F5 receptor (Y40/51)

Fig. 3. PDGFR-mediated survival of VSMC. A, the activated ChiR-WT protects VSMC against apoptosis to the same extent as the PDGF-BB-stimulated endogenous PDGFR. Cell death was induced by UV irradiation or H2O2 in the presence or absence of PDGF-BB or M-CSF. Apoptosis was analyzed by cell death detection ELISA. Data were measured in triplicate, normalized by the apoptosis rate of UV irradiation- or H2O2-treated cells in the absence of growth factors, and pooled data from at least three independent experiments are shown as means ± S.D. *, p < 0.05 versus UV irradiation alone or versus H2O2 alone. B, quantitative analysis of UV irradiation-induced apoptosis of VSMC expressing ChiR from the subtraction panel. Cell death was induced by UV irradiation in the presence or absence of M-CSF. Apoptosis was analyzed by cell death detection ELISA. Data were measured in duplicate and represent means ± S.D. from at least three independent experiments. Data were normalized by the apoptosis rate of UV light-irradiated cells in the absence of M-CSF for each mutant separately. The relative apoptosis rate for irradiated cells in the absence of M-CSF is 1 for each ChiR and indicated as ChiR. *, p < 0.05 versus ChiR-WT treated with UV irradiation in the presence of M-CSF. C, quantitative analysis of UV irradiation-induced apoptosis of VSMC expressing ChiR from the add-back panel. Cells were treated and analyzed as described in B. Data represent means ± S.D. from at least three independent experiments. *, p < 0.05 versus UV irradiation alone. D, analysis of PI3K-dependent effects on H2O2-induced apoptosis. Cells expressing ChiR-WT, F40/51, F5, or Y40/51 were starved and subsequently incubated with H2O2 in the presence or absence of M-CSF. Apoptosis was quantitatively analyzed by cell death detection ELISA. Data were measured in triplicates and normalized by the apoptosis rate of H2O2-treated cells in the absence of M-CSF for each mutant separately. Data represent means ± S.D. from at least three independent experiments, *, p < 0.05 versus H2O2 alone.
fully salvaged this cellular response (Fig. 3D).

Inhibition of Signaling Molecules by Pharmacological Inhibitors in Non-transfected Cells—A potential caveat of utilizing receptor mutants is the possibility that, in addition to the known binding partners of phosphoryrosines, other unknown signaling molecules may also interact with these sites. To confirm the results, which we obtained by the use of ChiR mutants, in a second approach, we repeated all measurements in PDGF-stimulated non-transfected VSMC in the presence of pharmacological inhibitors. Consistently, inhibition of PI3K by wortmannin completely suppressed the anti-apoptotic effect of PDGF-BB after UV irradiation, indicating that this signaling enzyme is involved in anti-apoptotic signaling, whereas inhibition of Src kinases (using SU6656), MEK1 (PD98059), and p38 MAPK (SB203580) had no effect on PDGF-dependent anti-apoptotic signaling (Fig. 4). Interestingly, inhibition of PLCγ by U-73122 also suppressed PDGF-mediated cell survival after UV irradiation but not when apoptosis was induced by H2O2. However, U-73122 appeared to affect cell survival by itself when used in higher concentrations (not shown). In summary, our data clearly demonstrate that anti-apoptotic signaling by the βPDGFR is mediated by only one of the receptor-associated signaling molecules, PI3K, whereas all other signal relay enzymes do not play a significant role.

PI3K/Akt Mediate Growth Factor-dependent Cell Survival via Phosphorylation of BAD and Forkhead Transcription Factors—Because activation of Akt is thought to play a critical role in PI3K-dependent regulation of apoptosis, we investigated the role of the receptor-associated proteins for PDGF-induced Akt activation. The ligand-induced phosphorylation of Akt was monitored by Western blot analysis using a phospho-specific Akt antibody in M-CSF-stimulated ChiR-expressing VSMC as well as in PDGF-stimulated non-transfected VSMC in the presence of specific inhibitors. Akt was maximally phosphorylated 5 min after PDGF stimulation and remained activated for several hours. In addition to Akt phosphorylation, its activity was monitored by the ability to phosphorylate its substrate GSK3β at serine 9 (Fig. 5A).

We next explored the contribution of each signaling molecule to βPDGFR-mediated Akt activation. In ChiR WT-expressing cells, Akt was phosphorylated upon M-CSF stimulation with a similar time course and to a comparable extent as in VSMC upon PDGF-BB-stimulation (Fig. 5B). The F40/51 receptor did not efficiently activate Akt, whereas the tyrosine to phenylalanine substitutions at all other sites had no effect on M-CSF-dependent activation of Akt. In the ChiR-F6-expressing cells, M-CSF-induced Akt activation was completely abolished (Fig. 5C). Restoration of the PI3K-binding site rescued M-CSF-dependent activation of Akt, whereas the F5, Y771, Y1009, and Y1021 mutants induced Akt activation to a much lesser extent. The fact that these mutants are still able to weakly phosphorylate Akt may be explained by weak binding of PI3K to these mutants (see Fig. 2E). Nevertheless, the Akt-substrate GSK3β is exclusively phosphorylated when PI3K efficiently binds to the activated receptor, underscoring that Akt is activated in response to PI3K binding to the ChiRs (Fig. 5, B and C). This result was confirmed with regard to the PDGF-stimulated endogenous PDGFR. Pharmacological inhibition of PI3K, but not of Src, PLCγ, MEK1 or p38, prevented PDGF-induced phosphorylation of Akt and GSK3β (Fig. 5D). These results indicate that only PI3K is able to efficiently activate the anti-apoptotic Akt pathway and suggest that the βPDGFR mediates its anti-apoptotic effect exclusively via the PI3K/Akt survival pathway.

To further investigate the signaling mechanisms downstream of PI3K/Akt, we evaluated the Akt-dependent inactivation of a set of pro-apoptotic proteins. To this end, we immu

![Image](https://via.placeholder.com/150)

**Fig. 4.** PDGF-BB inhibits UV irradiation- and H2O2-induced apoptosis of non-transfected VSMC in a PI3K-dependent manner. Cells were preincubated with the pharmacological inhibitors SU6656 (SU) (2.5 μmol/liter), wortmannin (W) (10 μmol/liter), U-73122 (U) (10 μmol/liter), PD98059 (PD) (10 μmol/liter), and SB203580 (SB) (10 μmol/liter) as indicated prior to induction of apoptosis via UV irradiation (A) or H2O2 treatment (B) and stimulation with PDGF-BB. Apoptosis was analyzed by cell death detection ELISA. Data were measured in triplicates and pooled. Data from at least three independent experiments are shown as means ± S.D. *, p < 0.05 versus UV irradiation alone or versus H2O2 alone.
DISCUSSION

In the present study we have systematically investigated the relative importance of the signaling enzymes that are recruited to the activated βPDGFR for protection against apoptosis in VSMC. Using receptor mutants and pharmacological inhibitors, we demonstrate that PDGF-dependent cell survival is mediated by only one of the βPDGFR-associated signaling molecules, PI3K. Although the other receptor-associated signaling

FIG. 5. Activation of Akt depends on PI3K binding. A, time-dependent activation of Akt in response to PDGF-BB. Starved VSMC were stimulated for the indicated times with PDGF-BB and probed with a phosphospecific Akt (Ser-473) antibody (middle lane) and an antibody against the phosphorilated Akt substrate GSK3β (bottom lane). Ras-GAP is shown as a loading control (top lane). B and C, activation of the Akt signaling pathway in Chir-expressing cells. Starved VSMC expressing the subtraction panel (B) or the add-back panel (C) of ChirRs were stimulated with M-CSF for 5 (+) or 30 min. Cell lysates were probed with a Ras-GAP antibody (top lane) and phosphospecific antibodies against Akt and GSK3β (bottom lanes). D, PDGF-mediated activation of Akt is PI3K-dependent. VSMC cultured in serum-free medium were treated for 5 min with PDGF-BB and the pharmacological inhibitors SU6656 (SU) (2.5 μmol/liter), wortmannin (W) (10 nmol/liter), U-75122 (U) (10 μmol/liter), PD98059 (PD) (10 μmol/liter), and SB203580 (SB) (10 μmol/liter) as indicated. Phosphorylation of Akt and GSK3β was analyzed as described above. The results presented are representative of at least three independent experiments.

FIG. 6. PDGFR-dependent phosphorylation of BAD and Forkhead transcription factors. A, time-dependent phosphorylation of BAD in response to PDGF-BB. Starved VSMC were stimulated with PDGF-BB for the indicated times. BAD was immunoprecipitated (IP) and probed with a BAD (top lane) and a phosphospecific BAD (Ser-136) antibody (bottom lane). B and C, BAD phosphorylation is mediated by PI3K. B, cells expressing Chir-WT, F40/51, or Y40/51 were starved and cells were stimulated for 10 min with M-CSF or left untreated. BAD was immunoprecipitated and probed with a BAD (top lane) and a phosphospecific BAD (Ser-136) antibody (bottom lane). C, VSMC cultured in serum-free medium were treated for 10 min with PDGF-BB and inhibitors as indicated. Immunoprecipitation of BAD was followed by Western blot analysis as described in A. D and E, phosphorylation of Forkhead transcription factors is mediated by PI3K. D, cells expressing Chir-WT, F40/51, F5, or Y40/51 were starved and cells were stimulated for 10 min with M-CSF or left untreated. Blots were probed with antibodies against Ras-GAP (top lane) and FKHR and FKHRL1 phosphorylated at Thr-24 or Thr-32, respectively (bottom lane). E, VSMC cultured in serum-free medium were treated for 10 min with PDGF-BB and inhibitors as indicated. Blots were probed as described in D. The results presented are representative of at least three independent experiments.
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molecules such as Src, PLCγ, RasGAP, and SHP-2 were previously shown to mediate survival signals, their contribution to growth factor-induced anti-apoptotic effects is not significant when compared with PI3K/Akt signaling. In addition, we characterized the signaling mechanisms downstream from PI3K/Akt, which includes phosphorylation/inactivation of pro-apoptotic BAD and Forkhead transcription factors (FKHRL1 and FKHR).

The complex methodology of ChiR mutants was used to reliably investigate the signaling pathways that contribute to cell survival in a relevant cell line that expresses endogenous PDGFR, namely VSMC. To circumvent endogenous PDGFR, the cytoplasmic domains of mutated βPDGFR were selectively stimulated via binding of M-CSF to the extracellular CSF1R domain. Using this highly efficient system we were able to selectively activate βPDGFR mutants in PDGFR-expressing cells and thereby prevent the ligand-induced activation of multiple signaling pathways without affecting their basal activity.

The systematic characterization of the “add-back” and “subtraction” panels of chimeric βPDGFR mutants as well as the use of pharmacological inhibitors consistently revealed that although the βPDGFR recruits multiple signaling enzymes, only PI3K is responsible for protection against apoptosis. Deletion of the PI3K-binding sites in the F40/51 receptor completely abolished the anti-apoptotic effect, whereas restoration of the PI3K-binding sites to the F5 receptor was fully sufficient to rescue βPDGFR-mediated cell survival. These data clearly demonstrate that binding and activation of PI3K to the βPDGFR is necessary and sufficient for βPDGFR-mediated suppression of apoptosis. Consistently, inhibition of PI3K with wortmannin led to a total loss of PDGF-BB-induced cell survival in non-transfected cells. In contrast, mutation of all other binding sites in the ChiR mutants as well as pharmacological inhibition of Src, PLCγ, or MAP kinases (ERK, p38) did not influence βPDGFR-mediated protection against apoptosis. Activation of the PI3K/Akt pathway has been shown to inhibit apoptosis in a variety of cell types (31–34). The evaluation of downstream signaling events revealed that Akt and the Akt effectors BAD, Forkhead transcription factors, and GSK3β were only phosphorylated when PI3K was active. Upon Akt-dependent phosphorylation at specific sites, pro-apoptotic proteins such as BAD, Forkhead transcription factors, and GSK3β are inactivated and sequestered in the cytosol bound to 14-3-3 (11, 35). Taken together, these findings indicate that growth factor-induced protection from apoptosis is exclusively mediated via PI3K, and the PI3K-dependent Akt signaling pathway leading to inactivation of pro-apoptotic proteins is crucial for cellular survival.

Based on previous work demonstrating that Src, RasGAP, SHP-2, and PLCγ all mediate survival signals (3–5, 14–20), it appears quite surprising that neither of them significantly contributed to growth factor-mediated cell survival in our system. The discrepancy between these studies and our data may be explained by the fact that these signaling enzymes under certain conditions exert anti-apoptotic effects indirectly by contributing to PI3K/Akt activation rather than by inducing independent survival signals, as indicated by some studies (14). When their relative contribution to growth factor-mediated survival signaling is compared with PI3K in one system, our data clearly show that of the βPDGFR-associated signaling molecules, only PI3K is sufficient to mediate an anti-apoptotic effect.

Our results on the role of PLCγ are inconsistent with a recent study reporting that PLCγ is required for the prevention of cell death in response to growth factors such as insulin-like growth factor-I (17). These authors investigated a special kind of cell death, anoikis, and it is likely that PLCγ-mediated signaling plays a limited role for cell survival under these special conditions. Our data obtained by the ChiR mutant system clearly indicate that PLCγ is not required for efficient growth factor-mediated cell survival (F1021 and Y1021 mutants). However, our findings obtained with the PLCγ inhibitor U-73122 are somewhat inconclusive, as pretreatment of non-transfected VSMC with U-73122 abolished the anti-apoptotic effect of PDGF in the case of UV irradiation-induced apoptosis, whereas it did not under the condition of oxidative stress. This discrepancy may be explained by differences in the experimental conditions. VSMC were incubated with U-73122 before and after UV irradiation because of one additional medium change after irradiation. In contrast, no medium change was necessary during H2O2-treatment. Thus, U-73122 may have accumulated within the cells after irradiation and in turn induced apoptosis by itself. Consistent with this idea, we observed that high doses of U-73122 induced cell death even in the absence of any other apoptosis inducer (not shown). When viewed together, our data strongly indicate that PLCγ has no significant function in anti-apoptotic growth factor signaling.

The Ras/Raf/MAPK pathway was shown to mediate cell survival during Drosophila development (36) and in the neuron system (15). Because neither deletion of the Grb2-binding site in the F6 mutant nor mutation of the RasGAP-binding site (F771) had any influence on cell survival, it is unlikely that Ras activation is involved in anti-apoptotic signaling in VSMC. Jung et al. (37) reported recently that ERK activation suppresses apoptosis in VSMC. Because the βPDGFR activates ERK via activation of PI3K, it is possible that PI3K-mediated ERK activation is required for cell survival. Our data demonstrated that co-incubation with the specific MEK-inhibitor PD98059 did not reverse PDGF-BB-mediated prevention of apoptosis. This suggests that protection from apoptosis by growth factors such as PDGF is at least in VSMC not ERK-dependent or demands a much higher degree of ERK activation. Jung et al. (37) incubated VSMC with fetal calf serum, which activates many types of receptors and thus provides excessive cross-talk. Thus, it seems that ERK activation is not sufficient to rescue cells from apoptosis and that co-activators derived from other pathways are necessary for full induction of ERK-dependent survival effects.

Another effector of the βPDGFR, STAT3, was discussed to protect from UV irradiation-induced apoptosis (5). In our model we cannot completely rule out anti-apoptotic effects of activated STAT3, because every ChiR mutant was able to activate STAT3 (not shown). However, a ChiR mutant, which mediated a stronger induction of STAT3 activity than the ChiR-WT (Y771, in which the RasGAP-binding site is restored in the F5 receptor), did not prevent UV irradiation- or H2O2-induced apoptosis indicating that STAT3 is at least not able to mediate anti-apoptotic effects in the absence of other signaling pathways. It is nevertheless possible that constitutively active or overexpressed STAT3 is capable of releasing survival effects.

Because physiological apoptosis inducers like tumor necrosis factor α have only little effect on VSMC apoptosis (38) we used two alternative approaches to induce apoptosis. UV irradiation was used as a general inducer of apoptosis, which acts by DNA damage and activation of death receptors (39) and is commonly used to induce apoptosis in various cell types. To rule out that our observations were limited to one specific apoptosis inducer, we additionally used a second unrelated approach, H2O2, which activates the mitochondrial death pathway to induce apoptosis in VSMC. This may even represent a more relevant approach, because large amounts of H2O2 are released from circulating macrophages during vascular conditions such as atherosclero-
sis, which influence the progression of these pathologies (40, 41). However, a potential disadvantage of \( \text{H}_2\text{O}_2 \) is its capability to activate many signaling pathways like PLC
\( \gamma \) (42). Because we obtained identical results with both apoptosis inducers, our findings indicate that growth factor-dependent activation of PI3K/Akt inhibits multiple death pathways.

In summary, the direct comparison of the various signaling pathways that are turned on in a PDGF-stimulated cell demonstrates that only one signaling enzyme recruited to the βPDGFR, PI3K, accounts for PDGFR-mediated cell survival, whereas all other receptor-associated signaling molecules do not play a significant role. Only PI3K is able to efficiently activate the anti-apoptotic Akt signaling pathway and mediates its survival effects via inactivation of pro-apoptotic proteins including BAD and Forkhead transcription factors.

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