p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway

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Clearance of fibrin through proteolytic degradation is a critical step of matrix remodeling that contributes to tissue repair in a variety of pathological conditions, such as stroke, atherosclerosis, and pulmonary disease. However, the molecular mechanisms that regulate fibrin deposition are not known. Here, we report that the p75 neurotrophin receptor (p75NTR), a TNF receptor superfamily member up-regulated after tissue injury, blocks fibrinolysis by down-regulating the serine protease, tissue plasminogen activator (tPA), and up-regulating plasminogen activator inhibitor-1 (PAI-1).

Introduction

Tissue scarring, characterized by cell activation, excessive deposition of ECM, and extravascular fibrin deposition, is considered a limiting factor for tissue repair. Fibrin, the major substrate of the serine protease plasmin, is a provisional matrix deposited after vascular injury (Bugge et al., 1996). The two plasminogen activators (PAs), namely tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), and their inhibitors, such as plasminogen activator inhibitor-1 (PAI-1), are key modulators of scar resolution by spatially and temporally regulating the conversion of plasminogen to plasmin resulting in fibrin degradation and ECM remodeling (Lijnen, 2001). In the peripheral nervous system, previous work by us and others showed that inhibition of fibrinolysis in mice deficient in plasminogen or tPA exacerbated axonal damage (Akassoglou et al., 2000) and impaired functional recovery after nerve injury (Siconolfi and Seeds, 2001). In accordance, mice deficient for fibrinogen showed increased regenerative capacity (Akassoglou et al., 2002). Studies of fibrin deposition in human diseases, in combination with experiments from mice deficient in plasminogen and PAs, have provided information about a wide range of physiological and pathological conditions that are exacerbated by defective fibrin degradation, such as wound healing, metastasis, atherosclerosis, lung ischemia, rheumatoid arthritis, muscle regeneration, and multiple sclerosis (MS) (Degen et al., 2001; Adams et al., 2004).
However, the molecular mechanisms that regulate proteolytic activity remain unclear.

In our current work, we focus on the mechanisms that regulate fibrinolysis after injury. Our previous studies demonstrated a correlation between fibrin deposition and expression of p75 neurotrophin receptor (p75NTR) after nerve injury (Akassoglou et al., 2002). Up-regulation of p75NTR is observed in MS (Dowling et al., 1992), stroke (Park et al., 2000), and spinal cord (Beattie et al., 2002) and sciatic nerve injury (Taniuchi et al., 1986), all of which are associated with fibrin deposition. p75NTR is also expressed in non-neuronal tissues (Lomen-Hoerth and Shooter, 1995) and is up-regulated in non-neuronal system diseases associated with defects in fibrin degradation, such as atherosclerosis (Wang et al., 2000), melanoma formation (Herrmann et al., 1993), lung inflammation (Renzi et al., 2004), and liver disease (Passino et al., 2007). p75NTR has been primarily characterized as a modulator of cell death (Wang et al., 2000) and differentiation (Passino et al., 2007) in non-neuronal tissues. The expression of p75NTR by cell types such as smooth muscle cells and hepatic stellate cells, which actively participate in tissue repair by migration, and secretion of ECM and extracellular proteases, raises the possibility for a functional role of p75NTR in disease pathogenesis that extends beyond apoptosis and differentiation.

We find that p75NTR is involved in the regulation of proteolytic activity and fibrin degradation. Mice deficient for p75NTR (Lee et al., 1992) show increased proteolytic activity and decreased fibrin deposition in two disease models: sciatic nerve injury and lung fibrosis. p75NTR regulates proteolytic activity by simultaneously down-regulating tPA and up-regulating PAI-1 via a novel cAMP/PKA pathway. p75NTR decreases cAMP via interaction with the cAMP-specific phosphodiesterase (PDE) isoform PDE4A4/5. This is of particular note, as selective PDE4 inhibitors have an anti-inflammatory action and have potential therapeutic utility in inflammatory lung disease, as well as in a wide range of neurologic diseases such as depression, spinal cord injury, MS, and stroke (Gretarsdottir et al., 2003; Nikulin et al., 2004; Houslay et al., 2005). Overall, the regulation of plasminogen activation by p75NTR identifies a novel pathogenic mechanism whereby p75NTR interacts with PDE4A4/5 to degrade cAMP and thus perpetuates scar formation that could possibly render the environment hostile for tissue repair.

**Results**

**Fibrin deposition is reduced in p75NTR−/− mice**

To examine whether p75NTR regulates fibrin deposition in the sciatic nerve we compared fibrin levels in wild-type (wt) and p75NTR−/− mice after injury. In wt mice, there is a dramatic increase of fibrin deposition (Fig. 1 c) and p75NTR expression (Fig. 1 d) after injury, when compared with uninjured nerves (Fig. 1, a and b). In contrast, p75NTR−/− mice show reduced fibrin deposition after injury (Fig. 1 e). Quantification of immunoblots reveals that p75NTR−/− mice have decreased fibrin by threefold 3 d and fourfold 8 d after injury (Fig. 1 g). Quantification of fibrin immunostaining also reveals that p75NTR−/− mice have significantly decreased fibrin (Fig. 1 h, P < 0.003). These results suggest that loss of p75NTR decreases the levels of fibrin in the sciatic nerve after injury.

**p75NTR regulates expression of tPA in the sciatic nerve after crush injury**

Analysis of total fibrinogen levels were similar in the plasma of wt and p75NTR−/− mice (unpublished data), suggesting the decrease in fibrin deposition is not the result of hypofibrinogenemia. Because fibrin removal depends on proteolytic activity (Bugge et al., 1996), we hypothesized that the decreased fibrin in the p75NTR−/− mice reflects an up-regulation of the proteolytic activity. p75NTR−/− mice have increased proteolytic activity (Fig. 2 b)

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**Figure 1.** Fibrin deposition is reduced in the sciatic nerve of p75NTR−/− mice. Immunohistochemistry for fibrin on uninjured wt [a] and 4 d after sciatic nerve crush injury wt [c] and p75NTR−/− mice [e]. Immunohistochemistry for p75NTR on uninjured wt [b] and 4 d after sciatic nerve crush injury wt [d] and p75NTR−/− mice [f]. Representative images are shown from n = 20 wt and n = 20 p75NTR−/− mice. (g) Western blot for p75NTR and fibrin on sciatic nerve extracts from uninjured wt, and wt and p75NTR−/− mice 3 and 8 d after injury. Myosin serves as loading control. Western blots were performed three times. A representative blot is shown. (h) Quantification of fibrin deposition shows significant decrease for fibrin in p75NTR−/− mice (n = 5), when compared with wt mice (n = 4). Bar graph represents means ± SEM (P < 0.003; by t test). Bar, 25 μm.
with the SC marker S100 (Fig. 2, k and l; green). tPA mice with knockout mice.
p75NTR is strongly activated by withdrawal of axons (Lemke and Chao, 1988) and its expression correlates with proliferating, non-myelin producing Schwann cells (SCs) (Zorick and Lemke, 1996). After sciatic nerve injury both p75NTR (Fig. 2 e, red) and tPA (Fig. 2 e, green) increase when compared with uninjured controls (Fig. 2 j), but show little colocalization (Fig. 2, e and h), suggesting that p75NTR-reexpressing SCs do not express tPA. Expression of tPA (Fig. 2 k, red) and p75NTR (Fig. 2 l, red) in SCs is confirmed using double immunofluorescence with the SC marker S100 (Fig. 2, k and l; green).

Genetic loss of tPA rescues the effects of p75NTR deficiency
To examine genetically whether the increased proteolytic activity in the p75NTR−/− mice was due to tPA, we crossed p75NTR−/− mice with tPA−/− mice and generated p75NTR−/−tPA−/− double-knockout mice. p75NTR−/− mice show a decrease in fibrin deposition (Fig. 3 b) and an increase in proteolytic activity (Fig. 3 f), compared with wt control mice (Fig. 3, a and e, respectively). In contrast, p75NTR−/−tPA−/− mice show increased fibrin deposition (Fig. 3 c) when compared with p75NTR−/− mice (Fig. 3 b) and no evidence of proteolytic activity (Fig. 3 g). As a control, tPA−/− mice also show no evidence of proteolytic activity after sciatic nerve crush injury (Fig. 3 h), as described previously (Akassoglou et al., 2000). Quantification of proteolytic activity is shown in Fig. 3 i. The evidence derived from the genetic depletion of tPA in the p75NTR−/− mice (p75NTR−/−tPA−/− mice, Fig. 3 g) are in accordance with the pharmacologic inhibition of tPA activity in the p75NTR−/− sciatic nerve using tPASTOP (Fig. 2 d). Overall, these results suggest that up-regulation of proteolytic activity in the sciatic nerve of p75NTR−/− mice is due to up-regulation of tPA.

p75NTR−/− SCs show increased expression of tPA and increased fibrinolysis
Because SCs are a major source for tPA after injury, we isolated primary SCs from wt and p75NTR−/− mice and cultured them on a three-dimensional (3D) fibrin gel. Wt SCs, which express high levels of p75NTR, form a monolayer on the fibrin gel (Fig. 4 a). In contrast, p75NTR−/− SCs degrade the fibrin gel (Fig. 4 b) and show a 2.7-fold increase of fibrin degradation (Fig. 4 c). p75NTR−/− SCs show a sixfold increase in tPA levels, when compared with wt SCs (Fig. 4 d; P < 0.01). These results suggest that p75NTR down-regulates tPA activity and blocks fibrin degradation in SCs in vitro.

Expression of p75NTR inhibits tPA and fibrinolysis
After finding a biological function for p75NTR in the regulation of tPA using SCs and sciatic nerves from p75NTR−/− mice, we used stable and transient transfections of p75NTR as well as

Figure 2. p75NTR regulates expression of tPA in the sciatic nerve after crush injury. In situ zymography in the presence of plasminogen on wt (a) and p75NTR−/− (b) mice and in the absence of plasminogen (c) or in the presence of plasminogen and tPASTOP (d) in p75NTR−/− mice. Arrows indicate the lytic zone. Double immunofluorescence for tPA (green) or p75NTR (red) on wt (e and h), p75NTR−/− (f and I) and p75NTR−/−tPA−/− mice (g). Uninjured wt sciatic nerve exhibits minimal proteolytic activity (f) and minimal tPA and p75NTR immunoreactivity (i). Zymographies have been performed on n = 10 wt and n = 10 p75NTR−/− mice. Representative images are shown. tPA (k) and p75NTR (l) expression in SCs was verified by double immunofluorescence with an S100 (SC marker) antibody. Arrows indicate double-positive cells (k and l, yellow). The experiment was repeated at two different time points (4 and 8 d after crush injury) in n = 4 mice per genotype per time point and representative images are shown. Bar: 400 μm (a–d, i), 150 μm (e–g, j), 20 μm (h, k, and l).
siRNA against p75NTR to test the properties of p75NTR in heterologous systems. To examine whether p75NTR could inhibit fibrin degradation, we first used NIH3T3 fibroblasts stably transfected with p75NTR that exhibit high levels of p75NTR (10^5 receptors/cell) (Hsu and Chao, 1993). NIH3T3 cells on a 3D fibrin gel degrade fibrin (Fig. 5 a), whereas NIH3T3p75NTR cells do not (Fig. 5 b). Expression of p75NTR inhibits fibrin degradation by 12-fold (Fig. 5 c; P < 0.001). NIH3T3 cells form lytic areas (Fig. 5 d), whereas NIH3T3p75NTR cells grow uniformly on fibrin (Fig. 5 e). NIH3T3 cells fully degrade the plasmin substrate casein (Fig. 5 f) but NIH3T3p75NTR cells do not degrade casein (Fig. 5 g), suggesting impaired proteolysis in NIH3T3p75NTR cells. Aprotinin, a general inhibitor of serine proteases, completely inhibits fibrin degradation by NIH3T3 cells (not depicted). In fibroblasts both tPA and uPA are involved in activation of plasminogen and fibrin degradation. tPA activity is significantly decreased in the NIH3T3p75NTR cells (Fig. 5 h). In contrast, expression of p75NTR has no effect on uPA activity (Fig. 5 i).

Tissue plasminogen activator (tPA) is a transcriptionally regulated immediate-early gene (Qian et al., 1993). Indeed, expression of p75NTR down-regulates tPA transcripts (Fig. 5 j). In addition, mRNA of PAI-1 is also up-regulated in NIH3T3p75NTR cells (Fig. 5 j). Real-time quantitative PCR shows a 10.1-fold decrease in tPA mRNA, a fourfold increase in PAI-1 mRNA, and a twofold decrease in uPA mRNA in NIH3T3p75NTR cells. Upon expression of p75NTR, the decrease of uPA RNA does not affect uPA activity (Fig. 5 i). In contrast, the decrease of tPA RNA in NIH3T3p75NTR cells results in a corresponding decrease in tPA activity (Fig. 5 h; P < 0.01).
After injury, sciatic nerves of p75NTR−/− mice show a fourfold increase of tPA RNA when compared with wt (Fig. 5 k). Moreover, p75NTR−/− mice show an increase in tPA RNA in primary cerebellar granule neurons (CGNs) (Fig. S1 c, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1), and increased proteolytic activity in the cerebellum (Fig. S1, a and b). Overall, these data suggest that expression of p75NTR inhibits the tPA/plasmin system both in vivo in the cerebellum and after sciatic nerve injury, as well as in vitro in primary neurons, SCs, as well as fibroblasts.

d5NTR regulates tPA and PAI-1 via a PDE4/cAMP/PKA pathway

Transcriptional regulation of tPA depends on the cAMP/PKA pathway (Medcalf et al., 1990). Indeed, elevation of cAMP, using dibutyryl-cAMP (db-cAMP), overcomes the inhibitory effect of p75NTR (Fig. 6 a). Moreover, cAMP elevation, elicited using the general PDE inhibitor IBMX, elevates tPA activity in NIH3T3p75NTR to the levels seen in NIH3T3 cells (Fig. 6 b). IBMX does not affect basal levels of tPA in NIH3T3 cells (Fig. 6 b). These data suggest that PDE activity is required for the p75NTR-induced tPA decrease.

PKA activity is decreased in NIH3T3p75NTR cells (Fig. 6 c, lanes 3 and 4) compared with NIH3T3 cells (Fig. 6 c, lanes 1 and 2), suggesting that p75NTR expression reduces PKA activity. KT5720, a specific PKA inhibitor, decreases tPA activity in NIH3T3 cells (Fig. 6 b). Because the cAMP/PKA pathway enhances tPA transcription and suppresses PAI-1 secretion (Santell and Levin, 1988), we tested whether the cAMP/PKA pathway influences the p75NTR regulation of tPA and PAI-1. Forskolin-induced cAMP elevation decreases, whereas KT5720-induced PKA inhibition decreases tPA RNA in NIH3T3 cells (Fig. 6 d). Forskolin treatment of NIH3T3p75NTR cells also increases both tPA RNA (Fig. 6 d) and activity (not depicted), whereas forskolin decreases PAI-1 RNA in both NIH3T3 and NIH3T3p75NTR cells (Fig. 6 e).

Similar to NIH3T3 cells, elevation of cAMP increases the activity of tPA in both wt and p75NTR−/− SCs (Fig. 6 f). Brain-derived neurotrophic factor (BDNF)/TrkB signaling has been shown to regulate tPA in primary cortical neurons (Fiumelli et al., 1999). In contrast to cortical neurons, SCs are known to express minute levels of TrkB but high levels of p75NTR (Cosgaya et al., 2002). We show here that treatment of SCs with either BDNF or nerve growth factor (NGF) has no effect on tPA (Fig. 6 f). Similar results are obtained after treatment of SCs with proNGF, the high-affinity ligand of p75NTR (Lee et al., 2001) (unpublished data). In addition, in NIH3T3 and NIH3T3p75NTR cells, which do not express Trk receptors, the p75NTR-mediated suppression of tPA activity occurs independent of neurotrophins or serum (unpublished data). In accordance, in NIH3T3 cells transient expression of the intracellular domain (ICD) of p75NTR decreases tPA similar to the full-length (FL) p75NTR (Fig. 6 g).
These data suggest that neurotrophin/p75NTR signaling is not involved in the regulation of tPA in SCs and fibroblasts and that regulation of tPA by p75NTR is independent of neurotrophins.

p75NTR decreases cAMP via PDE4

Because the effects of p75NTR were overcome by elevating cAMP, we examined whether p75NTR reduced cAMP levels. Indeed, cAMP is decreased 7.8-fold in NIH3T3p75NTR cells (Fig. 7 a; P < 0.0001). Transient expression of p75NTR in NIH3T3 cells decreases levels of cAMP (Fig. 7 b; P < 0.0005). Furthermore, siRNA knockdown against p75NTR leads to increased cAMP in both NIH3T3 and NIH3T3p75NTR cells (P < 0.0001). PKA activity assay shows increase of PKA in NIH3T3p75NTR cells. Forskolin increases cAMP mRNA in NIH3T3 and NIH3T3p75NTR cells. Inhibition of PKA by KT5720 decreases cAMP transcript. Quantification of PAI-1 mRNA changes by real time PCR shows a fourfold increase of PAI-1 mRNA in NIH3T3p75NTR cells compared with NIH3T3 cells. Forskolin increases cAMP activity in both wt [P < 0.001] and p75NTR−/− [P < 0.000001] SCs. NGF and BDNF do not affect activity of cAMP (P > 0.8 and P > 0.3, respectively). Transient overexpression of FL p75NTR or p75 ICD leads to decreased levels of cAMP in NIH3T3 cells. Experiments were performed at least 5 times in duplicates. *, P < 0.0001; **, P < 0.05; ***, P < 0.01. NS: non-significant. Bar graphs represent means ± SEM (statistics by ANOVA).

Figure 6. p75NTR regulates tPA and PAI-1 via a PDE4/cAMP/PKA pathway. (a) db-cAMP induces fibrinolysis in NIH3T3p75NTR cells. (b) IBMX increases tPA activity of NIH3T3p75NTR cells to the levels of NIH3T3 cells. Inhibition of PKA by KT5720 shows decrease of tPA activity in both NIH3T3 and NIH3T3p75NTR cells (P < 0.0001). (c) PKA activity assay shows decrease of PKA in NIH3T3p75NTR cells. (d) Forskolin increases cAMP mRNA in NIH3T3 and NIH3T3p75NTR cells. Inhibition of PKA by KT5720 decreases cAMP transcript. (e) Quantification of PAI-1 mRNA changes by real time PCR shows a fourfold increase of PAI-1 mRNA in NIH3T3p75NTR cells compared with NIH3T3 cells. (f) forskolin increases PKA activity in both wt [P < 0.001] and p75NTR−/− [P < 0.000001] SCs. NGF and BDNF do not affect activity of tPA (P > 0.8 and P > 0.3, respectively). (g) Transient overexpression of FL p75NTR or p75 ICD leads to decreased levels of tPA in NIH3T3 cells. Experiments were performed at least 5 times in duplicates. *, P < 0.0001; **, P < 0.05; ***, P < 0.01. NS: non-significant. Bar graphs represent means ± SEM (statistics by ANOVA).
p75NTR interacts with PDE4A4/5 and targets cAMP degradation to the membrane

Recruitment of PDE4 to subcellular structures such as the plasma membrane concentrates the activity of PDEs and reduces PKA activity by enhancing degradation of cAMP (Brunton, 2003; Houslay and Adams, 2003). We therefore examined whether p75NTR regulates cAMP via recruitment of PDE4. In NIH3T3p75NTR cells, p75NTR coimmunoprecipitates (co-IPs) with endogenous PDE4A (Fig. 8 a). No association is observed with the other three PDE4 sub-families, namely PDE4B, PDE4C, or PDE4D (unpublished data), suggesting that the effect was PDE4A specific. Based on the molecular weight of PDE4A at 109 kD, we determined that p75NTR co-IPs with the PDE4A5 isoform. Endogenous co-IP in CGNs (Fig. S3 a, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1) and in injured sciatic nerve (Fig S3 b) shows that p75NTR and PDE4A5 interact at endogenous expression levels. Analysis of lysates shows that the levels of PDE4A are similar in NIH3T3 and NIH3T3p75NTR cells (Fig. S3 c). These results show that p75NTR forms a complex with PDE4A5.

A functional consequence of the p75NTR–PDE4A5 interaction would be recruitment of PDE4A5 to the membrane resulting in decreased membrane-associated cAMP/PKA signaling.

To investigate whether p75NTR reduces membrane-associated PKA activity, we modified the genetically encoded A-kinase activity reporter, AKAR2 (Zhang et al., 2005) and generated pm-AKAR2.2, a membrane-targeted fluorescent reporter of PKA activity that generates a change in fluorescence resonance energy transfer (FRET) when it is phosphorylated by PKA in living cells (Fig. S4 a). As expected, NIH3T3 cells show a dramatic emission ratio change for the pm-AKAR2.2 in response to forskolin (Fig. 8 b). In contrast, NIH3T3p75NTR cells show an attenuated response, revealing reduced PKA activity at the plasma membrane (Fig. 8 b). Transient transfection of p75NTR confirmed the results observed in the stable NIH3T3p75NTR cells using the latest generation of plasma membrane–specific PKA biosensor AKAR3 (Allen and Zhang, 2006) (Fig. S4 b, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1). As expected, increased cAMP degradation at the plasma membrane results in decreased intracellular cAMP (Fig. S4 c; Fig. 7, a and b). Overall, our results showing reduced membrane-associated PKA activity upon expression of p75NTR suggest that p75NTR targets cAMP degradation to the membrane via its interaction with PDE4A5.

To verify the specificity of p75NTR–PDE4A5 association, a series of mapping studies were conducted using deletion mutants. PDE4A5 interacts with FL p75NTR, as well as deletions ∆3, ∆62,
Figure 8. p75NTR interacts with PDE4A4/5. (a) Endogenous PDE4A5 co-IPs with p75NTR in NIH3T3p75NTR cells. Lysates were immunoprecipitated with anti-p75NTR and probed with anti-PDE4A or anti-p75NTR. Due to the low endogenous levels of PDE4A, higher exposure was necessary to detect PDE4A5 in the lysates (see Fig. S3 c). (b) FRET emission ratio change of NIH3T3 and NIH3T3p75NTR cells for the pm-AKAR2.2 in response to forskolin. FRET change represents membrane activation of PKA (c) Mapping of the p75NTR sites required for interaction with PDE4A5. Schematic diagram of HA-tagged p75NTR intracellular deletions. TM, transmembrane domain; DD, death domain. Lysates were immunoprecipitated with an anti-HA antibody and probed with anti-PDE4A or anti-p75NTR. (d) Mapping of the PDE4A4 sites required for interaction with p75NTR. Schematic diagram of the C-terminal deletion of PDE4A4. Arrow indicates the deletion site. Lysates were immunoprecipitated with anti-p75NTR and probed with anti-PDE4A or anti-p75NTR. (e) Co-IP of purified, recombinant proteins reveals that both PDE4A4 and PDE4A5 interact with the ICD of p75NTR, but PDE4D3 does not. (f) PDE4A4 peptide library screened with recombinant GST-p75NTR ICD revealed three distinct domains of PDE4A4 (asterisks in d) that interact with the ICD of p75NTR, the LR1 domain (peptides 40 and 41), the catalytic domain (peptides 135 and 136) and the unique C-terminus (peptides 172 and 173). (g) Alanine scanning mutagenesis shows that substitution of C862 abolishes the interaction of p75NTR with the 173 peptide that is unique to PDE4A.
**p75NTR regulates plasminogen activation and fibrin deposition in a model of lipopolysaccharide-induced pulmonary fibrosis**

Because expression of p75NTR inhibits fibrinolysis in fibroblasts, we hypothesized that the role of p75NTR as a modulator of fibrinolysis extends to tissues outside of the nervous system that express p75NTR after injury or disease. Because p75NTR is expressed in the lung (Ricci et al., 2004), we compared the levels of fibrin in the lung of wt and p75NTR−/− mice in a model of lipopolysaccharide (LPS)-induced lung fibrosis (Chen et al., 2004).

LPS-treated wt mice showed widespread extravascular fibrin deposition (Fig. 9 b) and decreased proteolytic activity after LPS treatment (Fig. 9 e), when compared with saline-treated wt mice (Fig. 9 a and d). In contrast, p75NTR−/− mice show a 2.58-fold decrease of fibrin immunoreactivity (Fig. 9, c and j) and increased proteolytic activity (Fig. 9 f).

Decreased proteolytic activity in the lung after injury depends on the up-regulation of PAI-1 (Idell, 2003). Loss of PAI-1 protects from pulmonary fibrosis in LPS-induced airway disease, hyperoxia, and bleomycin-induced fibrosis (Savov et al., 2003). Because p75NTR increases PAI-1 (Fig. 5 j and Fig. 6 e), we examined whether p75NTR regulates expression of PAI-1 in vivo. PAI-1 is up-regulated in LPS-treated wt mice (Fig. 9 h) when compared with saline-treated wt mice (Fig. 9 g). In contrast, LPS-treated p75NTR−/− mice show similar immunoreactivity for PAI-1 (Fig. 9 i) as saline-treated wt mice (Fig. 9 g), suggesting that p75NTR induces up-regulation of PAI-1 after injury in the lung.

Western blots show a decrease in PAI-1 in the lungs of p75NTR−/− mice (Fig. 9 k). Similar to the p75NTR−/− mice, rolipram reduces fibrin deposition in the lung (Fig. S5, a and b; available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1) and sciatic nerve (Fig. S5, d–f), and decreases PAI-1 in the lung (Fig. S5 c), suggesting the involvement of PDE4 in p75NTR-mediated inhibition of fibrinolysis in vivo. Collectively, our data show that p75NTR increases fibrin deposition via a PDE4-mediated inhibition of plasminogen activation in both LPS-induced lung fibrosis and sciatic nerve crush injury. These data suggest a role for p75NTR/PDE4 signaling as a general regulator of plasminogen activation and fibrinolysis at sites of injury.

**Discussion**

Our study shows a novel direct interaction of p75NTR with PDE4A4/5, a specific PDE4 isoform, which results in the regulation of cAMP, a major intracellular signaling pathway, and mediates a major biological function of extracellular proteolysis and fibrinolysis (Fig. 10). p75NTR is expressed in a wide range of tissue injury models, where repair depends upon both cell differentiation and ECM remodeling. For example, we recently showed that in the absence of plasminogen the effects of p75NTR in tissue repair are protective due to its beneficial effects in cell differentiation (Passino et al., 2007). Similarly, in the flow-restricted carotid artery model of vascular injury that depends on uPA and not on tPA-mediated fibrinolysis (Kawasaki et al., 2001), p75NTR is protective due to the induction of smooth
that the interaction of p75 NTR with PDE4A4/5 could be poten-
tially mediated by direct binding to PDE4A domains, such as the C-terminal domain that is unique to this sub-family. In addi-
tion, in co-IP experiments we do not detect an interaction be-
tween p75NTR and β-arrestin (unpublished data). It is possible that the unique C-terminal domain of PDE4A could regulate isoform-specific PDE4 recruitment to subcellular locations. Biological roles have been described for PDE4D in ischemic stroke (Gretarsdottir et al., 2003) and heart failure (Lehnart et al., 2005) and for PDE4B in schizophrenia (Millar et al., 2005). Our study identifies a biological function for PDE4A4/5 as a molecular mediator of p75NTR/cAMP/ PKA signaling involved in the regulation of tPA and fibrinolysis.

We identify regulation of cAMP as a novel signaling me-
chanism downstream of p75NTR. Previous studies showed that β2-adrenergic receptors target degradation of cAMP to the membrane via recruitment of multiple PDE4 isoforms, such as PDE4B1, PDE4B2, and PDE4Ds (Perry et al., 2002). Our finding of interaction between p75NTR and PDE4A4/5 represents the first example of recruitment of a single PDE4 isoform to a transmembrane receptor. While interaction of β2-adrenergic recep-
tors with PDE4s is mediated via β-arrestin, our study suggests that the interaction of p75NTR with PDE4A4/5 could be poten-
tially mediated by direct binding to PDE4A domains, such as the C-terminal domain that is unique to this sub-family. In addi-
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In spinal cord injury in rodents, elevation of cAMP via the PDE4 inhibitor, rolipram, promotes axonal regeneration and functional recovery (Nikulina et al., 2004). In the sciatic nerve, reduction of cAMP after injury is attributed primarily to up-regulation of PDE4 by SCs (Walikonis and Poduslo, 1998). Based on our findings, it is possible that reexpression of p75NTR after injury could contribute to the activation of PDE4 and down-regulation of cAMP. BDNF, but not NGF, increases cAMP in neurons via TrkB (Gao et al., 2003). Moreover, BDNF/TrkB signaling overcomes the inhibition of nerve regeneration by myelin proteins via inhibition of PDE4 (Gao et al., 2003). We provide the first evidence for p75NTR in the regulation of cAMP by using genetic depletion, siRNA knockdown or up-regulation of the p75NTR. Our results suggest that p75NTR might exert the opposite function as Trk receptors by recruiting PDE4A4/5 and decreasing cAMP. Interestingly, PDE4A has been detected as the predominant PDE4 isoform at the cortico-
spinal tract (Cherry and Davis, 1999). Because p75NTR can act as a coreceptor for NogoR, a mediator of the inhibition of nerve regeneration, PDE4A interaction with p75NTR could play an inhibitory role in nerve regeneration by competing with neurotrophin signaling via Trk receptors.

It is possible that the increased expression of p75NTR by
eurons, glia, and brain endothelial cells could regulate the tem-
poral and spatial pattern of tPA expression during brain injury
or inflammation. p75NTR might also be upstream of other non-
fibrinolytic functions associated with tPA, such as neurodegener-
ation, synaptic plasticity, and long-term potentiation (Samson and Medcalf, 2006). Given the dependence of p75NTR func-
tions on the availability of ligands and coreceptors (Teng and Hempstead, 2004; Reichardt, 2006), further analysis will deter-
mine the role of p75NTR in extracellular proteolysis and ECM remodeling in different cellular systems. We show that expression of p75NTR can inhibit tPA in the absence of neurotrophin ligands. Constitutive expression of p75NTR may signal in a neurotrophin-independent manner to induce neuronal apoptosis (Rabizadeh et al., 1993), activation of Akt (Roux et al., 2001), and RhoGTPase (Yamashita et al., 1999). The regulation of cAMP identified here is an effect of expression of p75NTR that does not appear to depend on neurotrophin signaling. The cellular distribution of PDE4A4/5 would determine the involvement of p75NTR in the regulation of cAMP. It is possible that non-neurotrophin ligands that bind directly to p75NTR, such as β-amyloid (Teng and Hempstead, 2004), as well as the myelin/NogoR p75NTR–dependent inhibitors of nerve regeneration (Filbin, 2003), are able to regulate both cAMP and plasminogen activation by p75NTR. Because cAMP analogues decrease expression of p75NTR (Baron et al., 1997), it is possible that p75NTR by decreasing cAMP con-
tributes to the positive regulation of its expression. Because PKA phosphorlates p75NTR and regulates its translocation to lipid rafts (Higuchi et al., 2003), p75NTR via regulation of PKA might regulate its own subcellular localization. Given the multiple genes regulated by cAMP and PKA, other cellular functions may be regulated by p75NTR/cAMP signaling.
NGF/p75NTR signaling has been suggested to enhance local neurogenic inflammation to exacerbate pulmonary disease (Renz et al., 2004). Our study suggests an additional pathway for p75NTR as a regulator of expression of PAI-1 and a mediator of fibrosis. p75NTR in the lung is detected mainly in basal epithelial cells of bronchioles (unpublished data). Similar to p75NTR, PAI-1 is expressed by bronchial epithelial cells (Savov et al., 2003) and its expression results in an antifibrinolytic environment within the airway wall. Fibrin regulates both inflammation and airway remodeling (Idell, 2003; Savov et al., 2003). It is therefore possible that p75NTR-mediated regulation of PAI-1 via PDE4 could influence inflammatory and tissue repair processes in pulmonary disease. Although in chronic obstructive pulmonary disease the PDE4A4 isoform is specifically up-regulated (Barber et al., 2004) and considered a pharmacologic target (Idell, 2003; Savov et al., 2003), its expression results in an antifibrinolytic environment (Fabgennix). The co-IP buffer using NP-40 has been previously used to examine interactions of p75NTR with other intracellular proteins, such as TRAF-6 (Khrsugara et al., 1999) and PKA (Higuchi et al., 2003). For mapping experiments, PDE4A5 cDNA was cotransfected with HA-tagged p75NTR deletion constructs into HEK293 cells. IP was performed with an anti-HA antibody (Cell Signaling). Cells lysates were probed with an anti-PDE4A or an anti-p75NTR antibody (9651). For co-IP experiments using recombinant proteins, equimolar amounts (2 μM) of purified recombinant MBP-PDE4A5 (O’Connell et al., 1996), MBP-PDE4A4 (McPhee et al., 1999), MBP-PDE4D3 (Yarwood et al., 1999), and GST-p75NTR-ICD (Khrsugara et al., 2001) were mixed in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1 mM DTT, 0.5% Triton X-100, and 0.5% BSA) and incubated for 1 h at 4 °C. Washed glutathione-Sepharose beads were incubated for 1 h at 4 °C with 1 μg of each recombinant protein, washed, and analyzed. For co-IP experiments using antibodies, mice were injected with rolipram treatment after sciatic nerve injury, mice were injected with rolipram 5 mg/kg rolipram (Calbiochem). Induction with neurotrophins (Yamamoto and Loskutoff, 1996). Real-time PCR was performed using the Opticon DNA Engine 2 (MJ Research) and the Quantitect SYBR Green PCR kit (QIAGEN). Results were analyzed with Opticon 2 software using the comparative Ct method as described previously (Livak and Schmittgen, 2001). Data were expressed as ΔΔCt for the iPA gene normalized against GAPDH.

RT-PCR and real-time PCR
RT-PCR was performed as described previously (Akassoglou et al., 2002). Primers for iPA, uPA, and PAI-1 genes were used as described previously (Yamamoto and Loskutoff, 1996). Real-time PCR was performed using the Opticon DNA Engine 2 (MJ Research) and the Quantitect SYBR Green PCR kit (QIAGEN). Results were analyzed with Opticon 2 software using the comparative Ct method as described previously (Livak and Schmittgen, 2001). Data were expressed as ΔΔCt for the iPA gene normalized against GAPDH.

Quantification of iPA and uPA activity
Quantification of iPA and uPA activity in SC and fibroblast in lysates and supernatants was performed using the directional activity assay kits from American Diagnostica and Chemicon, respectively. To elevate cAMP cells were treated either with 2 mM db-cAMP (Sigma-Aldrich) or with 10 μM forskolin (Sigma-Aldrich) for 16 h. To block PKA activity, cells were treated with 200 nM KT5720 (Calbiochem). Induction with neurotrophins was performed using 100 ng/ml NGF and 50 ng/ml BDNF for 16 h before iPA assay.

Fibrin degradation assay
Coating with fibrin was prepared as described previously (Lansink et al., 1998). To quantitate fibrin degradation, the supernatant was aspirated and the remaining gel was weighed using an analytical balance. Decrease of gel weight corresponded to increased fibrin gel degradation.

Cell culture and transfections
Murine SCs were isolated as described previously (Syroid et al., 2000). NIH3T3 or HEK293 cells were cotransfected either with p75NTR FL, ICD and deletion constructs, and PDE4A5 cDNAs using Lipofectamine 2000 (Invitrogen) as described in the Results section. CGNs were isolated from P10 animals (Yamashita and Tohyama, 2003). CGNs were lysed immediately for co-IP, without pelleting. siRNA directed against p75NTR (Dharmacon) was transfection into SCs and NIH3T3p75NTR cells using Dharmafect (Dharmacon).

Materials and methods
Animals, sciatric nerve crush, and induction of lung fibrosis
p75NTR−/− mice (Lee et al., 1992) and iPA−/− mice (Carmeliet et al., 1994) were in C57Bl/6 background and purchased from The Jackson Laboratory. Double p75NTR−/− iPA−/− mice were generated by crossing p75NTR−/− mice (Ishii et al., 2004) and iPA−/− mice as controls. Sciatric nerve crush was performed as described previously (Akassoglou et al., 2000). Lung fibrosis was induced as described previously (Chen et al., 2004). For the rolipram treatments, mice were administered 5 mg/kg rolipram (Calbiochem) before the LPS injection as described previously (Miota et al., 1998). Mice were killed 4.5 h after LPS or saline administration. For rolipram treatment after sciatric nerve injury, mice were injected with rolipram (1 mg/kg) once daily for 8 d until tissue was harvested and processed for immunostaining.

Immunohistochemistry
Immunohistochemistry was performed as described in Akassoglou et al. (2002). Primary antibodies were sheep anti-human fibrinogen (1:200; US Biologicals), rabbit anti-human iPA (1:300; Molecular Innovations), rabbit anti-p75NTR clone 9651, (1:1,000), goat anti-p75NTR (1:200; Santa Cruz Biotechnology, Inc.), rabbit anti-mouse PAI-1 (1:500; a gift from David Loskutoff, Scripps Research Institute, La Jolla, CA), and mouse anti-S100 (1:200; Neomarkers). For immunofluorescence, secondary antibodies were anti-rabbit FITC and anti-goat Cy3 (1:200; Jackson Immunchemicals). Images were acquired with an Axiosplan II epifluorescence microscope (Carl Zeiss Microimaging, Inc.) using dry Plan-Neofluar lenses using 10× 0.3 NA, 20× 0.5 NA, or 40× 0.75 NA objectives equipped with Axiocam HRc digital camera and the Axiosview image analysis system.
μtein concentration was determined using the Bradford Assay (Bio-Rad Labo-

wheels (Lambda 10–2; Sutter Instrument Co.).

zymographies, cultures were washed four times with PBS/BSA and overlaid

μg/ml human plasminogen.

μM rolipram decreases fibrin deposition both in LPS-induced lung fibrosis

Statistics

Online supplemental material

Peptide array mapping

Peptide libraries were synthesized by automatic SPOT synthesis (Frank,

References


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trophin receptor is down-regulated by cyclic adenosine 3′,5′-monophos-

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