Antioxidants Relieve Phosphatase Inhibition and Reduce PDGF Signaling in Cultured VSMCs and in Restenosis

Kai Kappert, Jan Sparwel, Åsa Sandin, Alexander Seiler, Udo Siebolts, Olli Leppänen, Stephan Rosenkranz, Arne Östman

Objective—Growth factor– and reactive oxygen species (ROS)-induced activation of VSMCs is involved in vascular disease. This study investigates whether inhibitory oxidation of protein tyrosine phosphatases (PTPs) contributes to signaling in VSMCs in vitro and in vivo, and analyzes whether ROS- and growth factor–dependent vascular smooth muscle cell (VSMC) signaling is blunted by antioxidants that are able to activate oxidized PTPs.

Methods and Results—Signaling induced by H2O2 and platelet-derived growth factor (PDGF) was analyzed in VSMCs with or without the antioxidants N-acetyl-cysteine (NAC) and tempol. Effects of antioxidants on PDGF-stimulated chemotaxis and proliferation were determined. In vivo effects of antioxidants were analyzed in the rat carotid balloon-injury model, by analyzing neointima formation, cell proliferation, PDGF β-receptor status, and PTP expression and activity. NAC treatment prevented H2O2-induced PTP inhibition, and reduced H2O2- and ligand-induced PDGF β-receptor phosphorylation, PDGF-induced proliferation, and chemotaxis of VSMCs. Antioxidants inhibited neointima formation and reduced PDGF receptor phosphorylation in the neointima and also increased PTP activity.

Conclusion—PTP-inhibition was identified as an intrinsic component of H2O2- and PDGF-induced signaling in cultured VSMCs. The reduction in PDGF β-receptor phosphorylation in vivo, and the increase in PTP activity, by antioxidants indicate activation of oxidized PTPs as a previously unrecognized mechanism for the antirestenotic effects of antioxidants. The findings thus suggest, in general terms, reactivation of oxidized PTPs as a novel antirestenotic strategy. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: restenosis ■ VSMC ■ protein tyrosine phosphatase ■ platelet-derived growth factor ■ neointima formation

Vascular injury induces oxidative stress and elevated production of reactive oxygen species (ROS) in the vessel wall.1-3 Moreover, ROS are produced and act as second messengers as part of the signaling of receptor tyrosine kinases (RTKs) (reviewed in references 3, 4), which are activated after vascular injury. The most important ROS for pathological conditions are superoxide (O2-) and hydrogen peroxide (H2O2). Inhibition of ROS reduce vessel remodeling and restenosis.5 The underlying mechanisms remain incompletely understood.

PDGF β-receptor activation contributes significantly to vascular smooth muscle cell (VSMC) proliferation and migration, which is a hallmark of vascular diseases such as atherosclerosis and restenosis. PDGF ligands and receptors are significantly upregulated in atherosclerotic plaques. Moreover, PDGF β-receptor antagonists inhibit atherogenesis and restenosis in various models.6

PDGF β-receptors and other tyrosine kinases involved in VSMC proliferation and migration are regulated by protein tyrosine phosphatases (PTPs).7 PTPs which have been identified as negative regulators of PDGF β-receptors include the receptor-like PTP DEP-1, and the cytosolic phosphatases TC-PTP and PTP-1B (reviewed in reference 8). However, there are also indications that some PTPs, such as SHP-2, act as positive mediators of PDGF β-receptor signaling. The expression pattern of PTPs in VSMCs remains incompletely characterized. However, expression of PDGF receptor–antagonizing PTPs such as DEP-1, TC-PTP, and PTP-1B has been confirmed in previous studies.9

PTPs themselves are subject to multiple regulatory mechanisms. Reversible oxidation of the active site cysteine residue has been described as a general mechanism for negative regulation of PTPs.10 Oxidation of PTPs is therefore a candidate mechanism for ROS-mediated effects on VSMC proliferation and migration in vitro and in vivo.

This study investigates whether PTP-inactivation contributes to ROS signaling in VSMCs, and explores the possibility of antioxidant-mediated prevention of PTP-inhibition as a novel strategy for interference with restenosis.

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Materials and Methods
Detailed Materials and Methods are available as online supplement (please see http://atvb.ahajournals.org).

Primary Antibodies
Polyclonal rabbit antiserum against the PDGF β receptor was in-house derived, raised against a fusion protein composed of the C-terminal of the PDGF β-receptor and GST (CTβ). The other antibodies were derived from commercial sources (see online supplement).

Animals, Surgical Procedures, and Drug Treatment Protocol
Adult male Sprague-Dawley rats weighing 291 to 351 g (Mollegaard Breeding Center, Ry, Denmark) were used. Animal housing followed standard procedure. The experimental protocol was approved by the local Ethics Committee according to the European Union guidelines.

During inhalative isoflurane anesthesia balloon injury of the left common carotid artery was performed using a 2F atherectomy (V-Tech AB) catheter.11

The animals received no drug treatment (control), treatment with N-acetyl-cysteine (NAC, Sigma-Aldrich Sweden AB, Stockholm, Sweden), or 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol, Fluka Chemie GmbH, Buchs, Switzerland) (control n = 6, NAC n = 8, tempol n = 9). NAC (150 mg/kg bodyweight per day) was dissolved into the drinking water. Interindividual variations in blood concentrations were minimized by dosing once daily. Tempol (30 mg/kg bodyweight per day dissolved in 1 mL NaCl 0.9%) was administered via gavage once per day, and was kept protected from light until gavage. Drug treatment was started at the day of surgery and continued for 14 days until sacrifice of the animals.

Tissue Preparation
Fourteen days after angioplasty, the common carotid arteries were excised bilaterally and were divided into four parts. One part was OCT-embedded and snap-frozen in liquid nitrogen (LN2), the other six-μm sections of paraffin-embedded tissue were stained with hematoxylin-eosin. Three parts of the carotid vessel heights (proximal, middle, and distal parts) were sectioned and two sections per animals were quantified morphometrically for each height. Morphometric analysis of digital pictures was performed using NIH Image-J 1.41 software. For each arterial cross section the luminal, neointimal and medial area, and the intima-media ratio were calculated.

Histomorphometric Measurement
Six-μm sections of paraffin-embedded tissue were stained with hematoxylin-eosin. Three parts of the carotid vessel heights (proximal, middle, and distal parts) were sectioned and two sections per animals were quantified morphometrically for each height. Morphometric analysis of digital pictures was performed using NIH Image-J 1.41 software. For each arterial cross section the luminal, neointimal and medial area, and the intima-media ratio were calculated.

Immunohistochemistry
Antibody-staining and detection was performed as described.12 Primary antibodies (CTβ and ab16868) were incubated overnight at 4°C.

Staining intensity of ab16868 was quantified on an arbitrary scale between 1 to 10 by two investigators blinded to the treatment protocol. The quantifications of the two investigators of each individual section never varied more than 1 on the arbitrary scale. Additionally, sections were analyzed on a score according to protocol. The quantifications of the two investigators of each drug treatment were performed using standard procedures (see online supplement). Expression of analyzed genes was normalized to the expression of the house-keeping gene HPRT. Primer sequences are specified in supplemental Table I.

Cell Culture, Immunoprecipitation, and Immunoblotting
Rat vascular smooth muscle cells (VSMCs) were prepared from aortic arteries and used between passage 3 and 13.13 VSMCs were grown in DMEM supplemented with 10% FCS to subconfluence, synchronized by serum-deprivation (0.1% serum), and left resting or stimulated with PDGF-BB, H2O2 or with sodium vanadate (Sigma-Aldrich Sweden AB). Antioxidants were given to VSMCs for 60 minutes (NAC) or 30 minutes (tempol) before PDGF or H2O2 stimulation.

After rinsing twice in ice-cold PBS, cell lysis immunoblotting, visualization, and densitometric quantification was performed as described previously.14

Analysis of Proliferation, Chemotaxis, and Cell Viability
Analyses of DNA synthesis, chemotaxis, and cell viability was performed with commercial kits as detailed in online supplement. Calculations of three independent experiments were performed according to the manufacturer’s instructions. Cell viability for NAC was assessed by trypan blue staining because of interference of NAC with the LDH-assay kit. The assay was performed twice in duplicates and counted by at least two individuals, blinded to the treatment protocol.

Phosphatase Assay
VSMCs were synchronized by serum deprivation and werestimulated with 0.1 mmol/L H2O2 for 5 minutes. In assays with antioxidants, these were given to cells 60 minutes (NAC, 10 mmol/L) or 30 minutes (tempol, 3 mmol/L) before H2O2-treatment. Cells were lysed in buffer supplemented with 5 mmol/L NAC, but without Na2VO4. Thereafter, total cell lysate representing approximately 5000 cells were diluted in assay buffer, and phosphatase activity was determined by using a 32P-labeled peptide as substrate.15 For analyses of phosphatase activity in tissue, sections of injured carotid arteries derived from control-treated animals (300 μm/animal) and NAC- and tempol-treated animals (200 μm/animal) were pooled in lysis buffer without Na2VO4 according to treatment-groups. After adjustment for protein concentration, tissue lysates were subjected to phosphatase activity measurement. Assays were performed in duplicate, and phosphatase activity was expressed as the amount of 32P-labeled radioactivity released from the peptide after 7 minutes of incubation at 30°C.

Determination of Intracellular Peroxides
Intracellular peroxides were detected by flow cytometry. VSMCs (1×106 cells) were starved in serum-free medium for 6 hours and then loaded with CM-H2DCFDA (5-[and-6]-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, acetyl ester [Molecular Probes, C6027], 1 μmol/L, 15 minutes at 37°C). After treatment with or without N-acetylcysteine (10 mmol/L, 60 minutes) and Tempol (3 mmol/L, 30 minutes), cells were stimulated with H2O2 (1 mmol/L, 5 minutes). Cells were washed with ice cold PBS and detached from plates using trypsin. Cells were harvested by centrifugation (600g, 5 minutes) and washed again in PBS. Cells were excited with 488 nm UV line argon ion laser in a flow cytometer (BD FACSCalibur), and the DCF emission was recorded at 530 nm. Data were collected from at least 20 000 cells.

Statistics
Analysis of variance (ANOVA), and paired or unpaired t test were performed for statistical analysis, as appropriate. A probability value less than 0.05 was considered to be statistically significant. For morphometric analyses the mean of each animal at three different vessel heights were quantified and an unpaired t test was performed.
for statistical analysis. Data were expressed as mean±SEM or mean±SD, as indicated.

Results

H$_2$O$_2$ Induces Ligand-Independent and Antioxidant-Sensitive PDGF Signaling in VSMCs

Increased production of reactive oxygen species, such as H$_2$O$_2$, occurs under pathological circumstances in the vessel wall after eg, vascular injury by balloon-treatment. H$_2$O$_2$ has been shown to induce ligand-independent activation of RTKs. We therefore analyzed whether H$_2$O$_2$ was capable to induce ligand-independent activation of PDGF β-receptors and downstream signaling molecules in VSMCs.

VSMCs were subjected to H$_2$O$_2$ treatment. The concentration range was selected based on previous studies in which ROS-mediated PDGF receptor activation had been demonstrated. Phosphorylation of PDGF β-receptor, Akt, and MAPK p42/44 was induced by H$_2$O$_2$ treatment, and this could be blocked by NAC (supplemental Figure I). Antioxidants also decreased ligand-induced receptor phosphorylation (supplemental Figure II), suggesting that PDGF receptor phosphorylation in VSMCs is subject to negative control by PTPs subjected to oxidative regulation. The presence of PDGF receptor–antagonizing PTPs in VSMCs was also demonstrated by a strongly induced receptor phosphorylation following treatment with the general PTP inhibitor sodium-vandate (supplemental Figure I).

H$_2$O$_2$ Leads to PTP Inactivation in VSMCs, Which Can Be Restored by NAC

A large series of studies suggest that H$_2$O$_2$-mediated RTK activation involves inactivation of PTPs (reviewed in reference 3). We therefore analyzed whether H$_2$O$_2$ exposure of VSMCs was associated with a reduction in PTP activity.

Cells were exposed to 0.1 mmol/L H$_2$O$_2$ for 5 minutes. This concentration was selected based on previous studies on oxidation-induced PTP inactivation in fibroblasts which indicated partial PTP oxidation at this concentration. After H$_2$O$_2$ treatment, cells were lysed and total PTP activity was determined. This analysis revealed a significant reduction of PTP activity after H$_2$O$_2$ treatment, which could be prevented by pretreatment with NAC, but not tempol (Figure 1A). The ability of NAC to reduce cellular ROS levels, after H$_2$O$_2$-treatment was directly demonstrated by fluorescence measurements (Figure 1B). The same type of analyses also indicated a partial effect of tempol in H$_2$O$_2$-treated cells, although this effect was less prominent than that induced by NAC.

We thus conclude that H$_2$O$_2$-induced ligand-independent receptor phosphorylation is caused by inactivation of PDGF-receptor-targeting PTPs.

NAC and Tempol Reduce PDGF-Induced Receptor Phosphorylation, Proliferation, and Migration

Transient production of ROS, leading to PTP inactivation, has been described as an intrinsic part of RTK signaling (reviewed in 3). To explore whether this mechanism also operates in VSMCs, we analyzed the effects of antioxidants on PDGF stimulation of VSMCs.

PDGF stimulation induced robust proliferative and migratory responses in VSMCs. Interestingly, cotreatment with antioxidants significantly reduced these responses in a dose-dependent manner (Figure 2A and 2B). Cell viability was also analyzed with or without antioxidants and demonstrated clearly that antioxidants, at these concentrations, did not affect cell viability (Figure 2C).

Expression of DEP-1, PTP-1B, SHP-2, and TC-PTP was monitored by qRT-PCR analyses of control cells and cells cultured with NAC or Tempol. After 5 hours of treatment, a time point relevant for the migration assay, all PTPs were expressed in PTP-targeting VSMCs, we analyzed the effects of antioxidants on PDGF stimulation of VSMCs.
NAC-treated cells (data not shown), which indicates that modulation of PTP expression levels by antioxidants might contribute to the antiproliferative effect in vitro.

We thus conclude that inactivation of PTPs, through transient production of ROS, is a functionally significant aspect of PDGF β-receptor signaling in VSMCs, and that interference with this process, through treatment with antioxidants, represents a strategy for reducing PDGF-induced cell responses.

NAC and Tempol Inhibit Neointima Formation After Balloon Injury

Our in vitro experiments revealed that antioxidants potently inhibited H2O2-and PDGF-signaling, as well as migratory and proliferative responses in VSMCs through a mechanism involving reactivation of oxidized PTPs. This prompted analyses of the effects of antioxidants on restenosis and PDGF β-receptor phosphorylation in vivo, because both ROS and PDGF stimulation of VSMCs have been implied in this pathologic process.

NAC (150 mg/kg per day) and tempol (30 mg/kg per day) were administered on the day of balloon injury of the common carotid artery and daily for the following 14 days after injury. The neointimal area and intima-media-(I/M) ratio were significantly reduced in NAC- and tempol-treated animals compared with control rats (Figure 3A and 3B). In contrast, the medial area did not change in treated rats compared with the control group. Furthermore, in rats treated with NAC the levels of the proliferation-dependent transcript Ki67 were significantly lower compared with vehicle-treated animals (Figure 3C).

Together, these results clearly demonstrate that NAC and tempol inhibit neointima formation, through a mechanism that includes reduced proliferation of VSMCs.

NAC and Tempol Reduce PDGF β-Receptor Activation, but not Expression, In Vivo

The animal study was performed with the rationale that antioxidants might reactivate oxidized PTPs and thereby blunt ROS- and PDGF-induced VSMC proliferation and migration. To experimentally validate this hypothesis, restenotic lesions were analyzed to determine PDGF β-receptor status, ligand production, and PTP expression.

IHC revealed no altered expression of the PDGF β-receptor in vessel lesions after NAC- or tempol-treatment compared with vehicle-treated animals (Figure 4A, left). Transcript levels for the PDGF β-receptor were equal among the groups (Figure 4A, right). Furthermore, IHC and qRT-PCR analyses indicated similar levels of PDGF α-receptor expression in control- and NAC-treated animals, and a moderate reduction in the tempol-treated group (data not shown).

No significant differences in PDGF-B expression, or significant upregulations in the expression of PDGF receptor targeting PTPs, were observed as determined by qRT-PCR (Figure 4B).

Phosphorylation of the PDGF β-receptor in vivo was analyzed by IHC using an antibody recognizing pY1021 of the PDGF β-receptor (Figure 4C). Interestingly, a significant
reduction of staining in both NAC- and tempol-treated animals was observed in the intima (Figure 4C). Two different methods for quantification of pY1021 staining were used. As shown in Figure 4D, both methods revealed a significant reduction in the pY1021 staining in the intima, whereas no significant differences were observed in the adventitial and medial vessel layers. Decreased phosphorylation of PDGF receptor in antioxidant-treated animals was also indicated by an ELISA-based assay of PDGF receptor phosphorylation (data not shown).

Together, these analyses strongly suggest that activation of oxidized PDGF receptor-targeting PTPs contributes to the antirestenotic effects of NAC and tempol.

NAC and Tempol Increase PTP Activity in Vessels
To directly investigate whether treatment with antioxidants was associated with activation of oxidized PTPs, measurements of total PTP activity in vessel extracts was performed.

As shown in Figure 5, extracts from sections derived from antioxidant-treated animals displayed a significantly higher tyrosine phosphatase activity, as determined in an assay of in vitro dephosphorylation of 32P-labeled phospho-tyrosine peptides.

This experiment thus provides independent support for the notion that the reduction in PDGF receptor phosphorylation observed after treatment with NAC or tempol is caused by increased PTP activity.

Discussion
We demonstrate in this study that the antioxidant NAC reduces H2O2-mediated PTP inhibition in VSMCs (online supplement and Figure 1). The effects of H2O2 on PDGF receptor activation and on PTP activity occurred at similar concentrations of H2O2 (0.1 to 1 mmol/L). Furthermore, the antioxidants NAC and tempol, reduced PDGF-induced receptor phosphorylation, as well as migration and proliferation of VSMCs in vitro (online supplement and Figure 2). This implies that PTP inhibition is an intrinsic part of the PDGF-induced proliferative and migratory signaling. Finally, both antioxidants significantly reduced neointima formation (Figure 3). Importantly, the reduced neointima formation was paralleled by a reduction in receptor phosphorylation (Figure 4) and an increase in vessel PTP activity (Figure 5). These changes occurred in the absence of changes in expression levels of PDGF receptor, PDGF-B, and without upregulation of transcript levels of PDGF receptor targeting PTPs. This indicates that neointima reduction caused by antioxidants involved increased activity of PDGF receptor–antagonizing PTPs.

The findings of the present study thus provides experimental support for the two concepts that PTPs are endogenous antagonists of PDGF receptor signaling in VSMCs, and that this negative regulation of PDGF receptor signaling by PTPs is partially kept in check by inhibitory oxidation of receptor-antagonizing PTPs.

The strongest evidence for an involvement of PTPs in control of PDGF receptor signaling are derived from knockout studies, which have demonstrated enhanced PDGF receptor signaling in PTP1B and TC-PTP fibroblasts.14,18 In the case of TC-PTP depletion, a site-specific hyperphosphorylation of PDGF receptor was demonstrated, suggesting pathway-specific effects of individual...
PDGF β-receptor–targeting PTPs. In addition to these PTPs, SHP-1 and DEP-1 have also been implicated as negative regulators of PDGF β-receptor signaling.19,20 Although the pattern of PTP expression in VSMCs is incompletely characterized, it is noteworthy that expression of all four of these PTPs in VSMCs has been reported (reviewed in reference 8). Furthermore, downregulation of DEP-1 with siRNA enhances ligand-induced PDGF receptor phosphorylation and Erk activation, thus implying this particular PTP as one of the relevant PTPs in VSMCs (Kappert et al, unpublished data). The notion of an antagonistic effect of PTPs on PDGF-dependent restenosis was recently also supported by demonstration of an increase in neointima formation following adenoviral transfer of a dominant-negative version of PTP-1B.21

Concerning the importance of oxidative inhibition of PTPs for PDGF β-receptor signaling, it was shown already in 1995 that H2O2 production was a critical aspect of PDGF β-receptor signaling, although PTPs were not identified as the key targets at that time.17 More recently, demonstration of PTP oxidation on PDGF stimulation has been demonstrated in mouse and rat fibroblasts.10,22 These studies also demonstrated that PDGF β-receptor–induced PTP inhibition is PI3-kinase dependent and that PTP oxidation is restricted to PTPs in physical vicinity of PDGF β-receptors. The physiological significance of these events was recently supported by the demonstration that depletion of ROS-scavenging peroxiredoxin II enhances PDGF-receptor–induced proliferation, and concomitantly increases PDGF β-receptor phosphorylation and reduces PTP activity.23

These findings, and the observations of the present study, suggest a series of topics for continued studies. Highly prioritized is the identification of PTPs, which are the most important targets for PDGF β-receptor–induced ROS production, and investigations of the effects of antioxidants on different PDGF-receptor responses. It is also predicted that future studies on the details of ROS production and scavenging in VSMCs will reveal important insights.

NAC and tempol are both antioxidants, but belong to different antioxidant subgroups. NAC is the N-acetyl derivative of the protein amino acid L-cysteine and reacts with ROS such as H2O2.24 NAC serves also as a major precursor to the antioxidant glutathione. It is thought that NAC, in its role as a precursor to L-cysteine and glutathione, protects cell membranes against lipid peroxidation and protein oxidation.25 Tempol, in contrast, predominantly acts as a superoxide dismutase mimic and therefore acts upstream of H2O2 production.26 These described mechanisms of action are in general agreement with the present study which showed that tempol failed to affect phosphorylation induced by exogenous H2O2, but was almost equally potent as NAC in decreasing PDGF-induced responses.

Key findings of the present study are the novel demonstration that treatment with antioxidants reduces PDGF
β-receptor phosphorylation and increases PTP activity in neointima lesions. These findings merit special highlighting. Firstly, they suggest in general terms PTP reactivation as a previously unrecognized mechanism underlying the beneficial effect of antioxidants on neointima formation. Our study, focusing on PDGF receptors, are also compatible with inhibitory effects of antioxidants on other prorestenotic receptor tyrosine kinases, eg, the FGFR receptor which recently was shown to be negatively regulated by PTP-1B.22 Secondly, it provides evidence that PTP oxidation is a physiologically relevant mechanism for control of PDGF β-receptor signaling in vivo, particularly in restenosis. This notion has earlier only received experimental support from the demonstration of enhanced PDGF-dependent restenosis on depletion of peroxiredoxin II.23 Finally, and potentially most important, it suggests reactivation of oxidized PDGF β-receptor–targeting PTPs as a novel strategy for pharmacological interference with restenosis. Future studies will reveal whether this latter possibility will best be achieved through agents acting on oxidized PTPs, or with drugs modulating ROS levels.

Inhibitory effects of antioxidants or catalase on smooth muscle cell signaling and restenosis have been described earlier,26–30 and some suggestions on the underlying mechanism has been presented. These include demonstration of effects on apoptotic pathways as indicated by increased Bax expression and a reduced NF-kappaB activity,26,29 as well as reduced c-src activity.30 These changes could be secondary to reduced tyrosine kinase receptor activation. However, it is also possible that other mechanisms than PTP activation and concomitantly reduced tyrosine kinase activation contribute to antioxidant-mediated reduction in restenosis.

In summary, this study presents cell culture and in vivo experiments that demonstrate the possibility to interfere with PDGF β-receptor signaling in VSMCs by antioxidants that prevent PTP oxidation. It thereby suggests a novel molecular mechanism for the protective effect of antioxidants on neointima formation. In more general terms the study suggests activation of oxidized PTPs as a previously not recognized strategy for reducing vascular diseases associated with oxidative stress and/or dysregulated RTK-signaling. It is finally also predicted that future studies on the details of ROS production and scavenging in VSMCs will reveal yet unexplored options for novel treatments.

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Disclosures
None.

References


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Material and Methods

Antibodies
Monoclonal antibody PY99 (sc-7020) was from Santa Cruz (California, US), polyclonal antisera recognizing total Akt, Akt phosphorylated at Thr308, total MAP kinase p42/44, and p42/44 phosphorylated at Thr202/Tyr204 were obtained from Cell Signaling Technologies, Ltd. (Beverly, Massachusetts, USA). The polyclonal rabbit antibody ab16868 (Abcam, Cambridge, UK) recognize pY1021 in PDGF β-receptor.

qRT-PCR
RNA was isolated with the Arcturus PicoPure™ RNA Isolation kit (Arcturus Bioscience, Mountain View, California, USA), transcribed to cDNA using random primers and subjected to quantitative real-time PCR (qRT-PCR; SybrGreen Universal PCR Master Mix (Applied Biosystems, Foster City, USA)). The reaction was performed in triplicates with the ABI PRISM 7500HT real-time PCR cycler (Applied Biosystems).

Analysis of proliferation, chemotaxis and cell viability
DNA-synthesis induced by PDGF (20 ng/ml) was measured by a 5-bromodeoxyuridine (BrdU)-incorporation assay (Roche Diagnostics GmbH, Mannheim, Germany)\(^1\). Experiments were performed in quadruplicates and repeated at least three times. PDGF-dependent (10 ng/ml) chemotaxis was assayed using a 96-well modified Boyden chamber (ChemoTX cell migration microplate with a pore size of 8.0 µm (Neuroprobe, Gaithersburg, MD, USA)\(^2\). Experiments were carried out with 6 wells per condition and performed at least four times. Cell viability was determined measuring the lactate dehydrogenase (LDH) release from cells with the ‘CytoTox 96 Non-Radioactive Cytotoxicity Assay®’ kit (Promega, Mannheim, Germany)\(^3\).

References
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Table I: Primers used for quantitative real-time PCR
**Online Figure legends**

*Fig. I Ligand-independent induction of PDGF signaling is inhibited in the presence of N-acetyl-cysteine, but not tempol in VSMCs,*

Quiescent VSMCs were left resting (-) or were stimulated with H$_2$O$_2$ at indicated concentrations for 5 min (A), or were incubated with NAC (for 60 min) or tempol (for 30 min) prior to stimulation with 3mM (B) or 1mM (C) H$_2$O$_2$ for indicated time-periods. As a positive control for PDGF signaling quiescent cells were stimulated with PDGF-BB (PDGF) at 10ng/ml for 5 minutes (A). Treatments with combinations of different concentrations of PDGF and H$_2$O$_2$ were also performed (D). The effects on PDGF receptor phosphorylation by PTP inhibition by vanadate treatment (1mM, 60 min) were also analyzed (E). Total cell lysates were separated by SDS-PAGE, followed by standard blotting procedures. Membranes were incubated with antibodies as indicated.

*Fig. II Ligand-dependent PDGF β-receptor phosphorylation is inhibited by N-acetyl-cysteine and tempol in VSMCs*

A) Quiescent VSMCs were pretreated with NAC (10mM, 60 minutes) or tempol (3mM, 30 minutes), and were left resting (-) or stimulated with PDGF-BB (50ng/ml) for indicated time-periods. Membranes were incubated with antibodies as indicated. Shown is a representative blot of four separate experiments.

B) Densitometric analysis of the tyrosine-phosphorylation of the PDGF β-receptor induced by PDGF-BB, as shown in (A). Each value is the mean ± standard error of the mean from at least four separate experiments. Maximal phosphorylation of the PDGF β-receptor in control cells was set as 100%. *P<0.05 vs. control cells of the corresponding incubation time-period.
Supplemental Figure I A-C
### Table 1

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### Figure D

**IB:**
- **PDGF βR**
- **p-Tyr**

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### Figure E

**IB:**
- **PDGF βR**
- **p-Tyr**

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*Supplementary Figure I D-E*
Supplemental Figure II

A

IB:

PDGF βR

p-Tyr

PDGF-BB (min) - 5 15 30 5 15 30 5 15 30

NAC Tempol

B

PDGF β receptor phosphorylation (% of control)

0 20 40 60 80 100 120

PDGF-BB (min) - 5 15 30 5 15 30 5 15 30

NAC - - + - - + - - + -

Tempol - - - + - - + - - + -