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PRECLINICAL RESEARCH

The Tyrosine Phosphatase, SHP-1, Is a Negative Regulator of Endothelial Superoxide Formation

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OBJECTIVES We investigated the role of SH2-domain containing phosphatase-1 (SHP-1) in endothelial reduced nicotinamide adenine dinucleotide (phosphate) (NAD[P]H)-oxidase–dependent oxidant production.

BACKGROUND Superoxide ($O_2^{\cdot-}$) generation by endothelial NAD(P)H-oxidase promotes endothelial dysfunction and atherosclerosis. Signaling pathways that regulate NAD(P)H-oxidase activity are, however, poorly understood.

METHODS SH2-domain containing phosphatase-1 was inhibited using site-directed magnetofection of antisense oligodesoxynucleotides (AS-ODN) or short interfering ribonucleic acid (siRNA) in vitro in human umbilical vein endothelial cells (HUVEC) and in isolated hamster arteries; $O_2^{\cdot-}$ was measured by cytochrome c reduction in vitro. Activities of NAD(P)H-oxidase activity, phosphatidyl-inositol-3-kinase (PI3K), and SHP-1 were assessed by specific assays; Rac1 activation was assessed by a pull-down assay.

RESULTS Basal endothelial $O_2^{\cdot-}$ release was enhanced after inhibition of endothelial SHP-1 ($p < 0.01$), which could be prevented by specific inhibition of NAD(P)H-oxidase ($p < 0.01$); SHP-1 activity was high under basal conditions, further increased by vascular endothelial growth factor (10 ng/ml, $p < 0.05$), and abolished by SHP-1 AS-ODN treatment ($p < 0.01$), which also increased NAD(P)H-oxidase activity 3.3-fold ($p < 0.01$). Vascular endothelial growth factor also induced $O_2^{\cdot-}$ release ($p < 0.01$), which was even more enhanced when SHP-1 was knocked down ($p < 0.05$). The effect of SHP-1 was mediated by inhibition of PI3K/Rac1-dependent NAD(P)H-oxidase activation ($p < 0.01$); SHP-1 AS-ODN augmented tyrosine phosphorylation of the p85 regulatory subunit of PI3K ($p < 0.05$) and Rac1 activation. The latter was prevented by wortmannin, a blocker of PI3K.

CONCLUSIONS In HUVEC, SHP-1 counteracts basal and stimulated NAD(P)H-oxidase activity by negative regulation of PI3K-dependent Rac1 activation; SHP-1 thus seems to be an important part of endothelial antioxidative defense controlling the activity of the $O_2^{\cdot-}$-producing NAD(P)H-oxidase. (J Am Coll Cardiol 2005;45:1700–6) © 2005 by the American College of Cardiology Foundation

Reduced nicotinamide adenine dinucleotide (phosphate) (NAD[P]H)-oxidase–derived endothelial superoxide ($O_2^{\cdot-}$) production plays a decisive role in the development of atherosclerosis (1–4). Elevated $O_2^{\cdot-}$ levels activate proatherogenic signalling pathways and stimulate vascular smooth muscle cell proliferation (4,5), scavenge endothelium-derived nitric oxide, and, thus, aggravate endothelial dysfunction (2,4).

Activation of vascular NAD(P)H-oxidase contributes to atherosclerosis development (1,3,6), and expression of its subunits is increased in atherosclerotic plaques (7). The oxidase is constitutively active, but increases its activity upon stimulation more than 2-fold (8). The enzymatic complex consists of at least five subunits, among them the two membrane-spanning proteins Nox and $p22^{phox}$ and the cytosolic subunits $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, which, together with the small GTPase Rac1, associate with the membrane-bound subunits in order to form the active oxidase (8,9). At least two events seem to be necessary for assembly and full activation of the enzyme complex in endothelial cells. One is the translocation of the small GTPase Rac1 to the plasma membrane, an event that we have previously described to occur in endothelial cells (10). The second is a serine/threonin phosphorylation of $p47^{phox}$, which induces its membrane translocation (11).

Although signalling events downstream of NAD(P)H-oxidase–dependent $O_2^{\cdot-}$ production have been profoundly investigated, there is little evidence for regulating pathways leading to the enzyme's activation, particularly in endothelial cells. In addition to the serine/threonin phosphorylation leading to the activation of $p47^{phox}$ (11), tyrosine phosphorylation events seem to be needed for activation of the endothelial (10,12), smooth muscle cell (13,14), or platelet isoform (15) of the oxidase.

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Abbreviations and Acronyms

AS-ODN = antisense oligodesoxynucleotide
HUVEC = human umbilical vein endothelial cells
NAD(P)H = reduced nicotinamide adenine dinucleotide (phosphate)
$O_2^{\cdot-}$ = superoxide radical
PI3K = phosphatidyl-inositol-3-kinase
PTP = protein tyrosine phosphatase
AT = angiotensin
RIPA = radioimmunoprecipitation (buffer)
SHP-1 = SH2-domain containing tyrosine phosphatase-1
siRNA = short interfering ribonucleic acid
SS = sodium stibogluconate

In previous studies, we showed that tyrosine kinase blockers inhibit NAD(P)H-oxidase activity, whereas protein tyrosine phosphatase (PTP) inhibitors markedly increased it (16). In this context, we could demonstrate that angiotensin II (AT II) activates SH2-domain containing tyrosine phosphatase-1 (SHP-1) (also called PTN6, PTP-1C, SH-PTP-1) in endothelial cells via its AT-2 receptor subtype, which went along with decreased $O_2^{\cdot-}$ formation (17); SHP-1, which is preferentially studied in hematopoetic cells, is known to be a negative regulator of immune receptor signalling in lymphocytes, macrophages, and platelets, in which it is typically coactivated upon cellular stimulation to exert an autoinhibitory function (18–20).

In this study, we investigated whether endothelial cell SHP-1 is a negative regulator of endothelial NAD(P)H-oxidase dependent $O_2^{\cdot-}$ formation. Using the novel technique of magnetofection, we posttranscriptionally silenced SHP-1 using antisense oligodesoxynucleotides (AS-ODN) or short interfering ribonucleic acid (siRNA). We furthermore investigated which downstream-signalling targets are involved in SHP-1–dependent regulation of basal and agonist-induced NAD(P)H-oxidase activity.

METHODS

Endothelial cell culture. Primary human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by digestion with dispase (2.4 U/ml) and grown to confluence in medium 199 supplemented with 20% newborn calf serum and 20% endothelial growth medium (Promocell, Heidelberg, Germany) as previously described (HUVEC medium) (21).

Magnetofection of oligonucleotides. Single-stranded phosphorothioate AS-ODN or short interfering ribonucleic acids (siRNAs) were transfected using magnetofection, which was previously described as an advantageous technique for endothelial AS-ODN transfer (21,22). Assays were performed 24 h afterwards. Sequences of oligonucleotides were (5′-3′) cccttatttactactttcgc for random ODN and (5′-3′) ccttgagcagggtctctgcatcc for SHP-1 AS-ODN; the sequence of the double-stranded siRNA used was (only the sense strand is given in 5′-3′) guccguguugguugcagcutt, as control siRNA (5′-3′) uucuucgaacgugucacgutt was used. In some assays Cy3-fluorescence-labelled AS-ODN were used.

Measurement of superoxide radicals ($O_2^{\cdot-}$) and NAD(P)H-oxidase activity. For superoxide measurements and NADH-dependent $O_2^{\cdot-}$ formation in cell lysates, the cytochrome c reduction method was used as previously described (22). The $O_2^{\cdot-}$-dependent part of cytochrome c reduction was calculated from the difference in absorbance (550 nm) between samples incubated with or without superoxide dismutase (ϵ_{550nm} = 21.1/mol/cm).

Immunoprecipitation of SHP-1 and coimmunoprecipitation of p85 and phosphotyrosine. Human umbilical vein endothelial cells were washed, lysed in radioimmunoprecipitation (RIPA) buffer, and protein content determined (22). Aliquots containing 300 μg protein were incubated with 30 μl of protein A-containing Pansorbin beads (Calbiochem, Schwalbach, Germany) or protein G agarose for 1 h (4°C, gently shaking) to exclude nonspecific binding. Then samples were incubated with polyclonal primary anti-SHP-1 rabbit antibody (C-19) or monoclonal mouse p85 antibody (B-9, Santa Cruz Biotechnology, Heidelberg, Germany) overnight, followed by incubation with either Pansorbin beads for 2 h or protein G agarose (4°C, gentle shaking). After four washing steps using Tris-buffered saline and denaturation at 100°C (5 min), proteins were separated by SDS-PAGE using standard techniques (15). For coimmunoprecipitation experiments, membranes were first blotted using antiphosphotyrosine antibody (4G10, Upstate, Hamburg, Germany) followed by stripping (glycine 200 mmol/l, sodium chloride 500 mmol/l, pH 2.8) and counterstaining for confirming equal loading. Intensity of bands was quantified using a digital visualization system (Gel-Doc 1000, Bio-Rad, München, Germany).

SHP-1 activity assay. Equal amounts of SHP-1 were immunoprecipitated, collected on columns equipped with 10 μm filters (mobicols, MoBiTec, Göttingen, Germany), washed thoroughly using RIPA buffer, and equilibrated in the phosphatase assay buffer (Hepes 20 mmol/l, sodium chloride 100 mmol/l, magnesium chloride 5 mmol/l, manganese chloride 5 mmol/l, pH 6.5). After addition of 100 mmol/l p-nitrophenylphosphate, columns were incubated for 1 h (37°C), solutions transferred to multiwell plates, and extinction measured at 405 nm (SpectraFluor, Tecan, Germany).

Protein expression and Rac pull-down assay. BCL21-competent cells were transformed with plasmids (pGEX) containing cDNA coding for either GST-PAK or GST alone (control), grown on LB-ampicillin agarose dishes, and in LB-ampicillin medium using standard procedures. For protein induction, bacteria were grown to an optical density at 600 nm of 0.7 and induced at 37°C with 0.1 mmol/l isopropyl-1-thio-D-galactopyranoside and cultivated for 2 h. They were then lysed using NETN buffer (100 mmol/l NaCl, 1 mmol/l EDTA, 50 mmol/l Tris-HCl [pH 7.4],

JACC Vol. 45, No. 10, 2005
May 17, 2005:1700–6

0.5% Nonidet P-40, 1 mmol/l phenylmethylsulfonylfluoride, 5 mmol/l benzamidine), by addition of lysozyme (100 μg/ml), and by sonication, followed by centrifugation and pelleting. Fusion proteins contained in the supernatants were collected using glutathione-agarose beads (Amersham Biosciences, Braunschweig, Germany), washed, and the procedure controlled by SDS-PAGE. Pull-down assays were performed as previously described in detail (23).

Materials. Superoxide dismutase and dispase were from Roche (Penzberg, Germany). Antisense-ODN were from MWG Biotech, Ebersberg, Germany. CombiMAG particles were a kind gift of Dr. C. Bergemann (Chemicell, Berlin, Germany). Sodium stibogluconate (SS) was a kind gift of Dr. T. Yi (Cleveland, Ohio) (24). The peptides gp91ds-*tat* and scrambled-*tat* were kindly provided by Dr. P. Pagano (Detroit, Michigan). Effectene was from Qiagen, Hilden, Germany, and BCL21-competent cells were purchased from Invitrogen, Karlsruhe, Germany. All other substances were obtained from Sigma, Deisenhofen, Germany.

Statistical analysis. For descriptive purposes, all data are expressed as means ± SEM. Data were analyzed using one-way analysis of variance or Student *t* test. For paired experiments, the respective paired *t* tests were performed. Differences were considered significant when the error probability level was $p < 0.05$.

RESULTS

SHP-1 is constitutively active in endothelial cells and knocked out by antisense-magnetofection. Short-term magnetofection of SHP-1 AS-ODN (15 min) resulted in efficient deprivation of the SHP-1 protein after 24 h (n = 4) (Fig 1., inlet). This was associated with a loss of enzymatic activity of SHP-1 (n = 3, $p < 0.01$) (Fig. 1). Notably, there was relevant SHP-1 activity in cells grown under our experimental conditions.

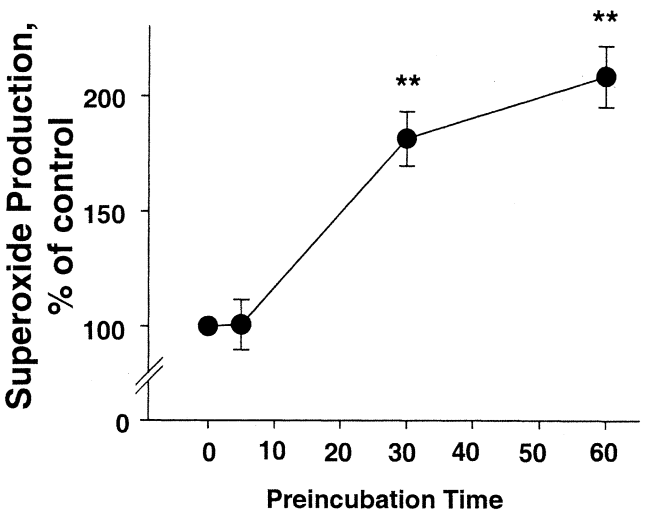

Figure 2. Pharmacological inhibition of SH2-domain containing tyrosine phosphatase-1 by sodium stibogluconate (SS) increases basal endothelial superoxide ($O_2^{\cdot-}$) production. Pretreatment with SS (10 μg/ml) significantly enhanced $O_2^{\cdot-}$ release from human umbilical vein endothelial cells monolayers. The effect of SS reached significant levels after 30 min of preincubation, indicating a nontranscriptional mechanism. **Significantly different vs. control at $p < 0.01$.

Inhibition of SHP-1 increases endothelial superoxide production. All means used to block SHP-1 in endothelial cells significantly increased basal $O_2^{\cdot-}$ release. Sodium stibogluconate, which specifically inhibits SHP-1 at concentrations of 10 μg/ml (24), increased $O_2^{\cdot-}$ release by 81.4 ± 1.5% (n = 32, $p < 0.01$) (Figs. 2 and 3). Increasing endothelial $O_2^{\cdot-}$ release by inhibiting SHP-1 activity appeared to be a nontranscriptional, rapidly occurring event, as 30 min of preincubation with SS (10 μg/ml) were sufficient to achieve the effect (Fig. 2, n = 12 for all time points).

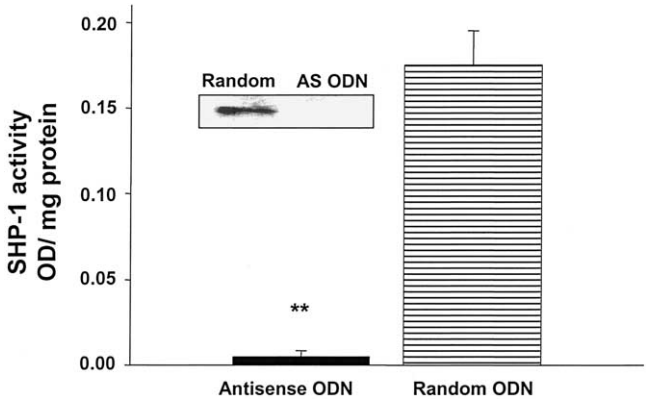

Figure 1. SH2-domain containing tyrosine phosphatase-1 (SHP-1) is constitutively active in endothelial cells and degraded by antisense-magnetofection. Magnetofection of antisense oligodesoxynucleotide (AS-ODN) against SHP-1 into human umbilical vein endothelial cells (HUVEC) resulted in deprivation of its protein associated with a loss of SHP-1 activity. Note that there is remarkable activity of SHP-1 in HUVEC grown under control conditions (random ODN, similar activity observed in untransfected cells, see Fig. 4). **Significantly different vs. control at $p < 0.01$.

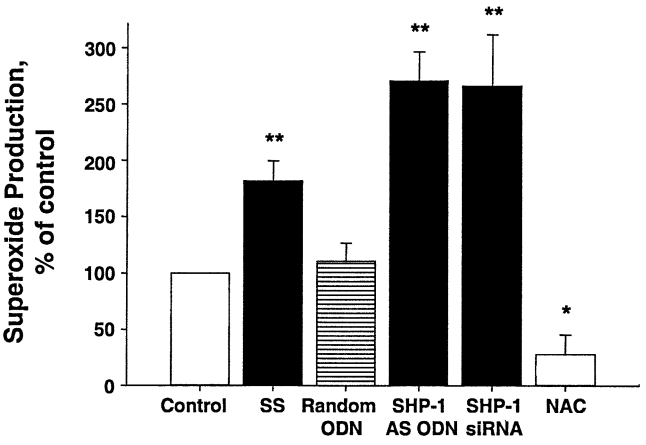

Figure 3. Degradation of SH2-domain containing tyrosine phosphatase-1 (SHP-1) protein increases endothelial $O_2^{\cdot-}$ production. Human umbilical vein endothelial cells $O_2^{\cdot-}$ release was enhanced, when SHP-1 was inhibited by a pharmacological agent (sodium stibogluconate [SS] 10 μg/ml) and when its protein was degraded by antisense oligodesoxynucleotide (AS-ODN) or short interfering ribonucleic acid (siRNA). *N*-Acetylcysteine (NAC) reduced $O_2^{\cdot-}$ production. *, **Significantly different vs. control at $p < 0.05$ and $p < 0.01$, respectively.

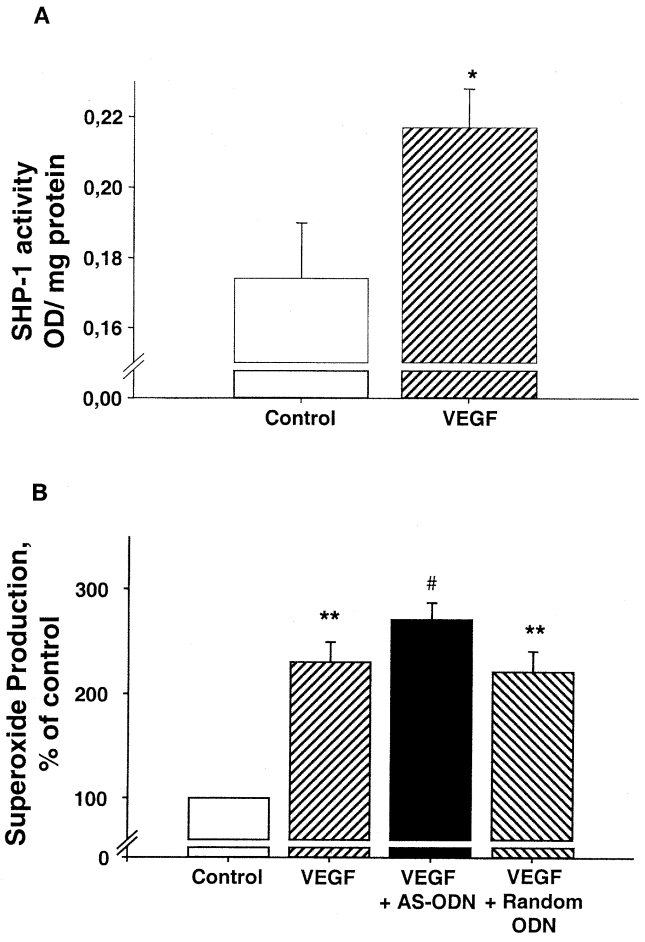

Figure 4. Influence of vascular endothelial growth factor (VEGF) on SH2-domain containing tyrosine phosphatase-1 (SHP-1) activity and superoxide production. **(A)** Stimulation with VEGF (10 ng/ml) enhanced basal phosphatase activity of SHP-1 in human umbilical vein endothelial cells (HUVEC). **(B)** VEGF (10 ng/ml) also increased HUVEC $O_2^{\cdot-}$ release, which was further enhanced when SHP-1 was knocked down using antisense oligodesoxynucleotide (AS-ODN). *, **Significantly different vs. control at $p < 0.05$ and $p < 0.01$, respectively; #significantly different vs. VEGF at $p < 0.05$.

Posttranscriptional deprivation of SHP-1 by AS-ODN treatment resulted in an even higher $O_2^{\cdot-}$ formation (by 170.4 ± 25%, n = 34, $p < 0.01$) (Fig. 3), while random ODN had no effect. Using RNA interference to knock out SHP-1 showed similar effects: magnetofection of 200 nmol/l of siRNA against SHP-1 increased endothelial $O_2^{\cdot-}$ release by 165.7 ± 45.5% (n = 8, $p < 0.01$) (Fig. 3). The antioxidant *N*-acetylcysteine (1 mmol/l, 30 min) completely blocked endothelial $O_2^{\cdot-}$ release (n = 3, $p < 0.05$) (Fig. 3).

To test, whether agonist-induced $O_2^{\cdot-}$ production was also influenced by inhibition of SHP-1, we applied VEGF (10 ng/ml, 30 min) to HUVEC, which not only increased enzymatic SHP-1 activity significantly (n = 5, $p < 0.05$) (Fig. 4A), but also $O_2^{\cdot-}$ release by 130.4 ± 19.2% (n = 9, $p < 0.01$) (Fig. 4B). When SHP-1 was knocked down using AS-ODN, this increase was further enhanced by another 18%, which was not the case when random ODN were used (n = 9 each, $p < 0.05$ vs. VEGF) (Fig. 4B).

SHP-1 down-regulates NAD(P)H-oxidase–dependent superoxide production. To identify the enzyme involved in $O_2^{\cdot-}$ production controlled by SHP-1, we pretreated HUVEC with various inhibitors of potential enzymatic sources for $O_2^{\cdot-}$. While inhibition of xanthine oxidase (oxypurinol, 300 μmol/l, n = 4), cyclooxygenase (indomethacin, 20 μmol/l, n = 3), or cytochrome P450 2C9 (sulfaphenazole, 50 μmol/l, n = 3) had no effect on SS-induced $O_2^{\cdot-}$ release (data not shown), specific inhibition of NAD(P)H-oxidase by the peptide gp91ds-*tat* abolished the effect of SHP-1 blockade (n = 9, $p < 0.01$) (Fig. 5A). Direct assessment of the NAD(P)H-oxidase activity by measuring NADH-dependent $O_2^{\cdot-}$ production in lysates of HUVEC confirmed the involvement of NAD(P)H-oxidase: the NAD(P)H-oxidase activity was unaffected in

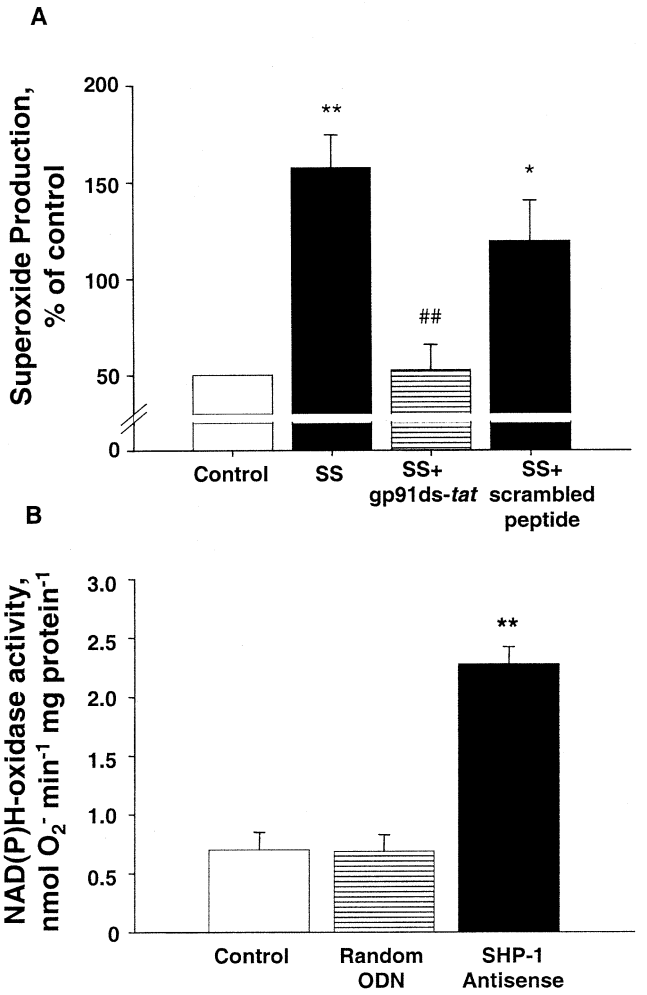

Figure 5. SH2-domain containing tyrosine phosphatase-1 (SHP-1) down-regulates reduced nicotinamide adenine dinucleotide (phosphate) (NAD[P]H)-oxidase activity. **(A)** Specific inhibitory peptides against NAD(P)H-oxidase (gp91ds-*tat*, 100 μmol/l) abrogated the increase in $O_2^{\cdot-}$ release in human umbilical vein endothelial cell (HUVEC) monolayers that was caused by sodium stibogluconate (SS). **(B)** NADH-dependent $O_2^{\cdot-}$ production in HUVEC protein lysates was increased 3.3-fold after treatment of cells with antisense oligodesoxynucleotide (AS-ODN) against SHP-1 but not with random ODN. *, **Significantly different vs. control at $p < 0.05$ and $p < 0.01$, respectively; ##significantly different vs. SS at $p < 0.01$.

JACC Vol. 45, No. 10, 2005
May 17, 2005:1700–6

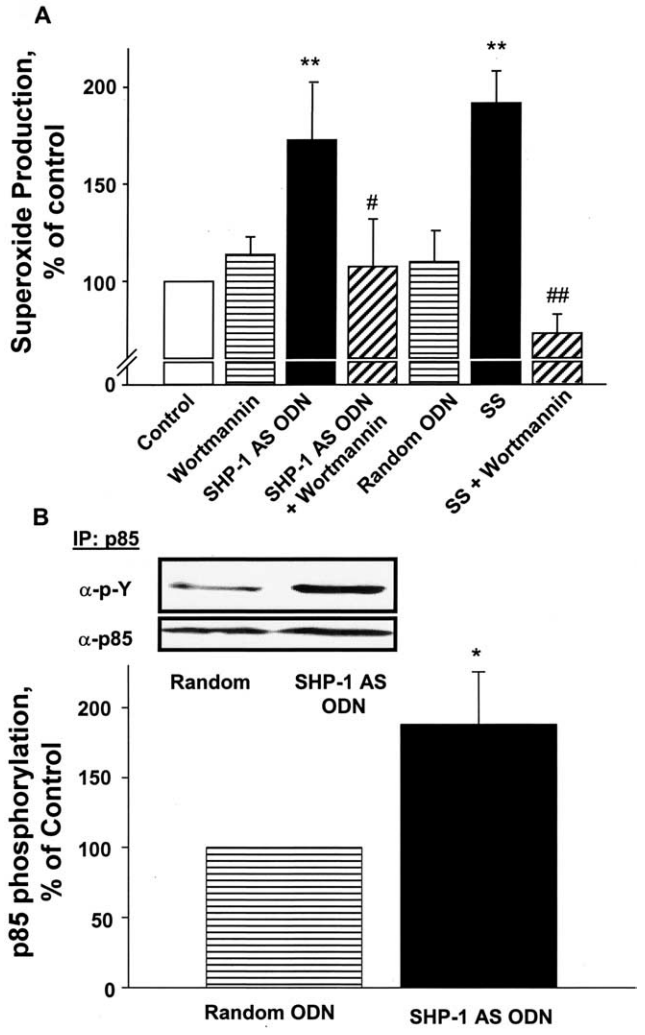

Figure 6. Phosphatidyl-inositol-3-kinase (PI3K) is a downstream target of endothelial SH2-domain containing tyrosine phosphatase-1 (SHP-1). **(A)** Inhibition of PI3K activity by wortmannin (10 nmol/l) abolished the increased $O_2^{\cdot-}$ production after treatment with SHP-1 antisense oligodesoxynucleotide (AS-ODN) or sodium stibogluconate (SS). **(B)** Co-staining of immunoprecipitates of the p85 regulatory subunit of PI3K showed increased tyrosine phosphorylation of p85 after knock out of SHP-1. Means of three independent experiments are shown. *, **Significantly different vs. random ODN at $p < 0.05$ or $p < 0.01$, respectively; #, ##significantly different vs. SHP-1 AS/SS at $p < 0.05$ or $p < 0.01$, respectively.

random ODN-treated cells, whereas it was 3.3-fold higher in cells pretreated with SHP-1 AS-ODN ($n = 36$, $p < 0.01$ vs. control and random) (Fig. 5B).

Phosphatidyl-inositol-3-kinase (PI3K) is a downstream target of endothelial SHP-1. Next, we studied whether PI3K is a target of SHP-1 in HUVEC. We treated HUVEC with an inhibitor of PI3K (wortmannin, 10 nmol/l, 30 min) in the presence or absence SHP-1 AS-ODN. Whereas the AS-ODN against SHP-1 induced the expected $O_2^{\cdot-}$ production, this was prevented when HUVEC were preincubated with wortmannin ($n = 4$, $p < 0.05$) (Fig. 6A), suggesting that SHP-1 under basal conditions down-regulates PI3K with subsequent attenuation in NAD(P)H-oxidase–dependent $O_2^{\cdot-}$ production. Similar observations were obtained in experiments using SS and wortmannin ($n = 9$, $p < 0.01$) (Fig. 6A). To confirm this hypothesis, we examined whether SHP-1 alters tyrosine phosphorylation of the p85 regulatory subunit of PI3-kinase. This subunit has previously been shown to inhibit PI3K activity when dephosphorylated (25,26). In fact, SHP-1 AS-ODN treatment increased tyrosine phosphorylation of p85 subunit by 88% compared to random ODN ($n = 6$, $p < 0.05$) (Fig. 6B).

SHP-1 down-regulates the NAD(P)H-oxidase component Rac1. Because Rac1 represents an essential component of NAD(P)H-oxidase, we studied whether SHP-1-dependent inhibition of NAD(P)H-oxidase was due to altered Rac1 activation. After SHP-1 AS-ODN treatment or SHP-1 inhibition by SS, there was a significant increase in Rac1 activation (pull-down assays, Fig. 7A), as evaluated by density measurements of GTP-bound Rac1 in comparison to total Rac1 ($n = 3$, Fig. 7B). Random ODN had no such effect. In accordance with $O_2^{\cdot-}$ measurements, Rac1 activation was prevented when AS-ODN-treated cells were incubated with wortmannin ($n = 3$, not shown), indicating that Rac1 is a downstream target of PI3K.

DISCUSSION

In this study, we report that SHP-1 is an important regulator of NAD(P)H-oxidase–dependent endothelial $O_2^{\cdot-}$ production. Our data suggest that SHP-1 is constitutively active in human endothelial cells and, thereby, controls basal NAD(P)H-oxidase activity and $O_2^{\cdot-}$ production.

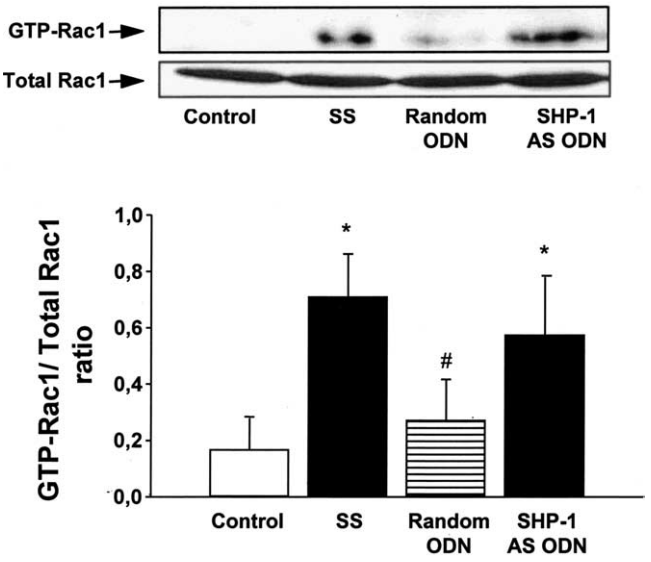

Figure 7. Inhibition of SH2-domain containing tyrosine phosphatase-1 (SHP-1) enhances Rac1 activation mediated by phosphatidyl-inositol-3-kinase (PI3K). **(Top)** Pull-down assays of Rac1 human umbilical vein endothelial cell revealed increased amounts of active Rac1 (bound to GST-PAK) after sodium stibogluconate (SS) or antisense oligodesoxynucleotide (AS-ODN) against SHP-1; SS-induced Rac1-activation was inhibited by wortmannin (not shown). **(Bottom)** The ratio between blot densities of GTP-bound Rac1 to total Rac1 revealed significant induction of Rac1 activity by SHP-1 AS-ODN and by SS (means of three experiments).

Upon endothelial cell stimulation, its activity can be further augmented. It then may serve as an autoinhibitory feedback mechanism to prevent excess $O_2^{\cdot-}$ release, as observed after stimulation with VEGF. We further show that SHP-1 inhibits NAD(P)H-oxidase by inhibition of PI3K activity and subsequent inactivation of the small GTPase Rac1.

Signalling events leading to endothelial NAD(P)H-oxidase activation are only partly characterized. In most cases, evidence is from studies in phagocytes. The latter express an NADPH-oxidase that is similar in structure, but differs in some features from endothelial NAD(P)H-oxidase (4,7,27,28). In comparison to the endothelial one, which uses Rac1, the phagocyte oxidase uses Rac2 (9). Another major difference is the isoform of the major large electron-transferring subunit, $gp91^{phox}$ (Nox2), which seems to be not expressed in all vascular cells. Endothelial cells, however, express it (28), but also the related isoforms Nox1, Nox3, and Nox4 (1). Data for signalling pathways involved in the activation of the vascular-type oxidase are rarer and often derived from studies in vascular smooth muscle cells (14,27), so the actual situation in endothelial cells remains uncertain. Nevertheless, it seems that protein kinase C activity leading to serine/threonin phosphorylation and subsequent translocation of $p47^{phox}$ (11) as well as translocation oft Rac1 (10) also take place during NAD(P)H-oxidase activation in endothelial cells.

Tyrosine phosphorylation events leading to vascular NAD(P)H-oxidase activation have been described by several groups including ours (10,12,14,16,29). In addition, we have observed that nonspecific PTP inhibitors dramatically increase $O_2^{\cdot-}$ release from endothelial cells (16). This suggested that PTP exerts inhibitory function when they are active; SHP-1 is a PTP expressed in endothelial cells (17), which is well-known to exert autoinhibitory action in B- and T-lymphocytes (18,19,30,31). Interestingly, neutrophils from SHP-1–deficient mice have inappropriate activation patterns associated with an increased oxidant production (30). In our study, endothelial SHP-1 controls phosphorylation of p85, the regulatory subunit of PI3K. It is known that tyrosine phosphorylation of p85 activates PI3K (26). In our experiments, inhibition of PI3K prevented the enhanced $O_2^{\cdot-}$ release caused by SHP-1 AS-ODN. Conversely, SHP-1 AS-ODN enhanced tyrosine phosphorylation of p85. Both inhibition of SHP-1 by SS and by AS-ODN furthermore increased Rac1 activity, which was not observed when PI3K was inhibited. This suggests that Rac1 is downstream of PI3K, which itself is regulated by SHP-1. Thus, inhibition of PI3K by dephosphorylation of p85 is the mechanism by which SHP-1 inhibits endothelial NAD(P)H-oxidase. Involvement of PI3K in NAD(P)H-oxidase activation has been suggested after observations in smooth muscle cells, where $O_2^{\cdot-}$ release was inhibited by wortmannin, but the underlying mechanisms remained unclear (14,32). We now observe that during SHP-1–dependent NAD(P)H-oxidase activation, Rac1 is activated in a PI3K-dependent manner, which unveils the mechanism by which SHP-1 regulates NAD(P)H-oxidase activity. Recently, Li and Shah (33) suggested that the active enzymatic complex of NAD(P)H-oxidase is preassembled in endothelial cells, where the enzyme exhibits constitutive activity. In view of these data, our findings suggest that a high turnover of Rac1, which is regulated by PI3K, could represent a rapid molecular switch to further regulate the activity of endothelial NAD(P)H-oxidase. In accordance with this hypothesis, enhanced NAD(P)H-oxidase activity after blockade of SHP-1 was quickly reversed by treatment of cells with a PI3K inhibitor in our study.

We conclude that SHP-1 is an important regulator of $O_2^{\cdot-}$ generation by endothelial NAD(P)H-oxidase under basal conditions or after stimulation with VEGF. It remains to be shown whether other stimuli that increase endothelial $O_2^{\cdot-}$ release, such as AT II or shear stress, also do so or even act by down-regulating the activity of SHP-1. According to our findings, any signalling that impairs endothelial SHP-1 activity could promote the development of endothelial dysfunction by enhanced $O_2^{\cdot-}$ formation. Thus, decreased activity of SHP-1 may be an important contributor to atherosclerotic disease. On the other hand, because direct intervention studies using nonspecific antioxidants failed to show beneficial effects (34), enhancing or preserving SHP-1 function in endothelial cells might represent an interesting tool to counteract increased oxidative stress within the vascular wall in cardiovascular disease.

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