Improvement of Peripheral Endothelial Dysfunction by Protein Tyrosine Phosphatase Inhibitors in Heart Failure

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Background—Chronic heart failure (CHF) induces endothelial dysfunction characterized by a decrease in nitric oxide (NO) production in response to flow (flow-mediated dilatation [FMD]). Because activation of endothelial NO synthase (eNOS) by flow requires tyrosine phosphorylation, we tested whether endothelial dysfunction could be corrected by increasing phosphotyrosine levels using protein tyrosine phosphatase (PTP) inhibitors and especially inhibitors of PTP1B.

Methods and Results—CHF was induced by coronary ligation in mice, and FMD was assessed in isolated and cannulated mesenteric artery segments (2 mm in length and <300 μm in diameter). CHF almost abolished FMD but only moderately affected the response to acetylcholine. In mice with CHF, the PTP1B inhibitors AS279, AS098, and AS713 restored FMD to levels similar to those of normal mice. This restoration was reduced by inhibitors of eNOS and phosphatidylinositol-3 kinase. Polymerase chain reaction and Western blot showed that arteries express PTP1B, and this expression was not affected by CHF. Immunolocalization revealed the presence of PTP1B in the endothelium and the adventitia. Flow induced a transient eNOS phosphorylation that was absent in CHF. PTP1B inhibition stimulated early eNOS phosphorylation and increased phosphorylation of Akt.

Conclusions—Our results demonstrate for the first time that PTP1B inhibitors may be potent treatments for endothelial dysfunction. (Circulation. 2006;114:2498-2507.)

Key Words: acetylcholine ■ blood flow ■ echocardiography ■ endothelium ■ endothelium-derived factors ■ heart failure ■ signal transduction

Endothelial production of nitric oxide (NO) is markedly affected early in cardiovascular diseases and in the presence of various cardiovascular risk factors. Although many factors may contribute to the decreased NO availability in diseases, one possibility that has not been widely evaluated in terms of pathophysiology or of therapeutic target is the possible role of impaired transduction pathways leading to endothelial NO synthase (eNOS) activation, especially the possible role of impaired phosphorylation pathways.

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Indeed, the “physiological,” calcium-independent1–3 activation of eNOS by shear stress exerted by intraluminal flow (which represents the major stimulus for the permanent endothelial release of NO in the circulation)4,5 requires tyrosine phosphorylation,6,7 together with activation of phosphatidylinositol-3 kinase (PI3K)/Akt and eNOS phosphorylation.8–10

The role of tyrosine phosphorylation in the activation of eNOS raises the hypothesis that endogenous stimulation of this enzyme may be achieved in pathological situations of impaired NO production by increasing tyrosine phosphorylation, especially through the use of protein tyrosine phosphatase (PTP) inhibitors, and thus that PTP inhibitors may be treatments of endothelial dysfunction.

Chronic heart failure (CHF) induces endothelial dysfunction of peripheral resistance arteries,11,12 and this most likely contributes to the increased peripheral resistance observed in this disease.13 The resulting increased afterload may then aggravate heart failure and favor cardiac decompensation. This suggests that interventions that restore endothelial function and especially NO production in heart failure might exert beneficial effects in this disease.

In previous experiments, we found that CHF markedly reduces NO-mediated, flow-dependent vasodilatation of peripheral arteries, whereas the vasodilatory response to acetylcholine is largely maintained.11,12,14 A possible explanation for this differential alteration would be that CHF selectively impairs the calcium-independent phosphorylation pathways by which shear stress (but not acetylcholine) activates eNOS and thus that manipulation of these phosphorylation pathways might restore NO production in CHF.
The present study was thus designed to assess whether a short-term increase in tyrosine phosphorylation restores endothelial function in CHF. For this purpose, we used inhibitors of PTPs and especially of PTP1B.

Methods

Induction of Heart Failure
CHF was induced by myocardial infarction of the left ventricle. For this purpose, 6-week-old male C57BL6 mice (Charles River, Saint Aubin Les Elbeuf, France) were anesthetized with a mix of ketamine (90 mg · kg⁻¹; Merial, France) and xylazine (3.6 mg · kg⁻¹; Bayer, France) and ventilated, and a thoracotomy was performed. The left main coronary artery was ligated close to its origin. Postinfarction mortality in this mouse model is ~30% to 40%, primarily occurring around days 4 to 7 after myocardial infarction. Sham-operated mice (control) were subjected to the same protocol except that the artery was not ligated.

Hemodynamic Parameters Assessed in Anesthetized Mice
After 2 months, mice were anesthetized with a mix of ketamine and xylazine. The right carotid artery was cannulated with a micromanometer (1.2F; Millar Instruments, Houston, Tex) advanced into the aorta to record arterial pressure. The micromanometer was then advanced into the left ventricle (LV) to record LV pressure and its derivative. After 2 months, mice were anesthetized and subjected to a laparotomy. A portion of the mesenteric artery, 2 mm around days 4 to 7 after myocardial infarction. Sham-operated mice (control) were subjected to the same protocol except that the artery was not ligated.

Mesenteric Resistance Artery Preparation and Functional Vascular Studies
Two months after coronary ligation, the mice were anesthetized and subjected to a laparotomy. A portion of the mesenteric artery, 2 mm in length and <300 μm in diameter, was carefully isolated under a dissection microscope transferred to an arteriograph system (Living Systems Instrumentation, Burlington, Vt). The artery was mounted on 2 glass micropipettes and pressurized to 60 mm Hg. The artery segment was bathed in an organ chamber containing a physiological salt solution with the following composition (mmol/L): NaCl 118.3, KC1 4.7, CaCl₂ 2.7, NaHCO₃ 25, MgSO₄ 1.2, glucose 5.5. Flow-mediated dilatation (FMD), ie, changes in vessel diameter in response to stepwise increases in intraluminal flow (0 to 200 μL/min), was evaluated after preconstriction by phenylephrine (10⁻⁶ mol/L).

Inhibitory Effects and Selectivity of the PTP1B Inhibitors
Catalytic domains for human PTPs were cloned and expressed as previously described. The PTPs were tested at 75 ng/mL in a buffer that contained 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L MgCl₂, 0.01% vol/vol IGEPAL CA-630 (Sigma; L-3021), 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (Sigma; E-7889), and 6,8-difluoro-4-methylumbelliferyl phosphate (DifMUP; Molecular Probes, Carlsbad, Calif) at 100 μmol/L. The inhibitors were dissolved in dimethyl sulfoxide (final dimethyl sulfoxide concentration 5%). Plates were incubated 30 minutes at 37°C and read for fluorescence at Ex355 nm/Emission 460 nm (Wallac Victor). The final DifMUP concentration was 5 (PTP1B, T-cell protein tyrosine phosphatase), 20 (PTP-β, density-enhanced phosphatase, leukocyte common antigen-related phosphatase, SHP2), or 30 μmol/L (glomerular epithelial protein 1, PTP-κ, PTP-μ, and vacuolin H1-related).

Polymerase Chain Reaction Experiments
The expression of PTP1B mRNA in the mesenteric arteries was assessed by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) with a light cycler (Roche, Basel, Switzerland). For this purpose, 4 segments of mesenteric arteries were isolated from each animal (either normal or CHF) and pooled before RNA extraction. This procedure yielded to isolated RNA of 500 to 900 ng mRNA. The primers were obtained from Proligo Primers and Probes and had the following sequences: PTP1B: sense 5'-CATCCAGACTGCCGACCA-3'; antisense 5'-ATGATGAACCTTGCCCTG-3'; 18S: sense 5'-TGGAGCGATTGCTGTTGTT-3'; antisense 5'-CGCTGACCCAGTCAGTAG-3'.

Immunohistochemistry
Localization of PTP1B was assessed by immunohistochemistry in transverse sections of formalin-fixed mesenteric arteries. Sections (4 μm) were incubated for 1 hour with an anti-PTP1B antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) followed by incubation with a biotin-labeled donkey anti-goat antibody (Amersham Bioscience, Uppsala, Sweden). Sections were subsequently incubated for 30 minutes with streptavidin–horseradish peroxidase conjugate

### TABLE 1. Selectivity of PTP Inhibitors Used in This Study

<table>
<thead>
<tr>
<th>PTP1B</th>
<th>TC-PTP</th>
<th>PTP-β</th>
<th>DEP-1</th>
<th>LAR</th>
<th>SHP1</th>
<th>SHP2</th>
<th>GLEPP-1</th>
<th>PTP-κ</th>
<th>PTP-μ</th>
<th>VHR</th>
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<tbody>
<tr>
<td>AS279</td>
<td>0.30</td>
<td>0.75</td>
<td>3.78</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1.24</td>
<td>0.95</td>
<td>0.46</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>AS098</td>
<td>0.24</td>
<td>0.94</td>
<td>4.51</td>
<td>38</td>
<td>&gt;40</td>
<td>0.40</td>
<td>0.34</td>
<td>0.07</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>AS713</td>
<td>0.14</td>
<td>0.37</td>
<td>14.50</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>8.73</td>
<td>7.74</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
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</table>

TC-PTP indicates T-cell protein tyrosine phosphatase; DEP-1, density-enhanced phosphatase 1; LAR, leukocyte common antigen-related phosphatase; GLEPP-1, glomerular epithelial protein 1; and VHR, vacuolin H1-related.
(Amersham, Uppsala, Sweden) and revealed with 5-aminon-9-ethy carbazol (Sigma) for 15 to 20 minutes.

**Western Blot**

Arteries from normal and CHF mice were mounted on the arterio graph system, preconstricted by phenylephrine, and subjected to 200 μL/min flow in the absence or presence of AS279 (10^{-7} mol/L) as described previously for functional studies. At different times after application of flow, the arteries were quickly taken out of the perfusion apparatus, immediately frozen in liquid nitrogen, and then stored at −80°C for later analysis. One individual perfused artery served only for a single protein assay, except for eNOS and phosphorylated eNOS (P-eNOS), in which 2 arteries were pooled for each experimental condition to perform analysis of eNOS and P-eNOS in parallel blots. Arteries were homogenized in cold buffer containing sodium dodecyl sulfate (1%), Tris-HCl (10 mmol/L, pH 7.4), aprotinin (5 μg/mL), leupeptin (2.5 μg/mL), and sodium orthovanadate (1 mmol/L). The amount of proteins loaded on the gel was verified by a Bradford assay and was in each case between 35 and 40 μg. Samples used for the measurement of eNOS or P-eNOS were immunoprecipitated with protein AAG plus agarose (vol/vol; Santa Cruz Biotechnology, Inc). Immunoprecipitated proteins or proteins from the homogenized tissue were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Mini Gel Protein III System, Bio-Rad Laboratories, Hercules, Calif) and transferred on Hybond ECL membranes (Amersham Biosciences) for 45 minutes at 100 V (Mini trans-blot Cell, Bio-Rad Laboratories). Membranes were incubated with the following primary antibodies; anti-PTP1B (polyclonal; Santa Cruz), anti-eNOS (monoclonal; Transduction Laboratories), anti-Akt (polyclonal; Cell Signaling Technology, Danvers, Mass), anti-phospho-Akt (P-Akt; polyclonal; Santa Cruz Biotechnology, Inc), anti–phospho-Akt (P-Akt; polyclonal; Santa Cruz Biotechnology, Inc), anti–P-eNOS (monoclonal; Cell Signaling Technology, Danvers, Mass), anti-eNOS (monoclonal; Serina 1177; Serva, Heidelberg, Germany), and anti–P-eNOS (monoclonal; Serina 473; Santa Cruz), and anti–P-eNOS (monoclonal; Serina 1177; Serva, Heidelberg, Germany). Membranes were washed again and incubated with horseradish peroxidase–conjugated secondary antibodies (for monoclonal antibodies: donkey anti-mouse; for polyclonal antibodies: goat anti-rabbit; both from Jackson Immunoresearch Laboratories, West Grove, Pa). Proteins were then visualized with the use of a Chemiluminescence kit (Lumi Light, Roche).

**Cell Culture**

Rat coronary endothelial cells were isolated as described previously.9 Briefly, hearts were isolated from Wistar rats (Charles River, Saint Aubin Les Elbeuf, France), and the coronary bed was perfused in a retrograde manner through the aorta with a solution of EBS (Sigma) containing sodium citrate (Sigma), collagenase (Roche), and trypsin (Cambrex). Released endothelial cells were then grown on fibronectin-coated (Becton Dickinson, Franklin lakes, NJ) cultured flasks in EGM-2-MV Bullet Kit medium (Cambrex). Western blot analysis of PTP1B was performed from confluent cells at passage 3.

**Statistical Analysis**

All data are presented as mean±SEM. Differences between groups were analyzed by Student t test (for hemodynamic data and PCR) or 2-factor repeated-measures ANOVA, with flow as one factor and treatment as the other factor, with the use of SigmaStat version 3.5. A probability value <0.05 was considered statistically significant. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Inhibitory Effects and Selectivity of the PTP1B Inhibitors**

To evaluate the role of PTPs in CHF in a pharmacologically relevant context, we used recently developed organic PTP inhibitors from a medicinal chemistry program aimed at discovering PTP1B inhibitors. Two chemically distinct compounds (AS279 and AS098) were first selected on the basis of the fact that they show relative selectivity toward PTP1B (Table 1) and are cell permeable (data not shown). Because those compounds also inhibit SHP2, which appears to also contribute to shear-stress–mediated NO production,16,17 we selected 1 additional compound, AS713, which shows little inhibitory activity on SHP2 at the concentration tested (1 μmol/L; IC_{50} 0.14 and 8.7 μmol/L for PTP1B and SHP2, respectively; Table 1).

**Characterization of Heart Failure in Mice**

Two months after coronary ligation, infarct size averaged 45±3% of the LV (n=8). Table 2 shows that, after 2 months, coronary ligation induced significant increases in LV end-diastolic and end-systolic diameters, together with significant decreases in LV fractional shortening and cardiac output. Invasive measurements of LV pressure (Table 2) show that coronary ligation decreased LV dP/dt_{max} and dP/dt_{min}.

**Functional Vascular Studies**

**Effects of CHF on the Response to Flow and to Acetylcholine**

In normal mice, stepwise increases in intraluminal flow induced a progressive increase in artery diameter (FMD). CHF abolished FMD of mice mesenteric arteries (Figure 1, right; dilatation at 2500 Circulation

**TABLE 2. LV Hemodynamic and Echocardiographic Parameters**

<table>
<thead>
<tr>
<th></th>
<th>Normal (Sham-Operated)</th>
<th>CHF Mice (n=11)</th>
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<tbody>
<tr>
<td>LV dP/dt_{max}, mm Hg</td>
<td>7003±390</td>
<td>5510±304*</td>
</tr>
<tr>
<td>LV dP/dt_{min}, mm Hg</td>
<td>5470±274</td>
<td>4402±235*</td>
</tr>
<tr>
<td>LV end-diastolic diameter, cm</td>
<td>0.31±0.01</td>
<td>0.58±0.04*</td>
</tr>
<tr>
<td>LV end-systolic diameter, cm</td>
<td>0.13±0.01</td>
<td>0.49±0.04*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>60±3</td>
<td>17±3</td>
</tr>
<tr>
<td>Cardiac output, mL · min^−1</td>
<td>112±5</td>
<td>82±3*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.01 by t test.

**Effects of Nonselective PTP Inhibition on the Response to Flow**

In mesenteric arteries isolated from normal mice, in vitro administration of the nonselective PTP inhibitor sodium orthovanadate (10^{-5} mol/L) did not affect FMD (Figure 2, left). In contrast, in arteries isolated from CHF mice, orthovanadate restored FMD to a level not significantly different from that observed in normal mice (Figure 1, left).

**Effects of Selective PTP1B Inhibitors on the Response to Flow**

The effects of 3 chemically distinct selective inhibitors of PTP1B AS279 (10^{-7} mol/L), AS098 (10^{-5} mol/L), and AS713 (10^{-8} mol/L) on the response to flow in arteries from normal or CHF mice are shown in Figures 3 to 5. In arteries isolated from normal mice, the PTP1B inhibitors did not significantly affect FMD, although AS279 and AS713 tended to reduce it, and this was significant for AS279.

In contrast, all 3 inhibitors markedly and significantly increased FMD in arteries isolated from CHF mice. In the
case of AS279, this increase appeared concentration dependent because the improvement was also found at $10^{-6}$ mol/L (data not shown). The highest concentration of AS279 restored FMD to levels similar to those of normal mice.

**Role of PI3K and NO in FMD in Normal Mice**

The effects of the PI3K inhibitor wortmannin ($10^{-6}$ mol/L) and the NOS inhibitor L-NA ($10^{-5}$ mol/L) on FMD in arteries isolated from normal mice are shown in Figure 6. Both inhibitors markedly reduced FMD.

**Role of PI3K and NO in the Restored FMD Induced by PTP1B Inhibitors**

The effects of the PI3K inhibitor wortmannin ($10^{-6}$ mol/L) and the NOS inhibitor L-NA ($10^{-5}$ mol/L) on FMD in arteries

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**Figure 1.** Effect of CHF on the vasodilatory response to acetylcholine (left) and flow (right). Concentration-response curves were performed in isolated, pressurized, and preconstricted mesenteric arteries taken from normal (sham-operated) mice (open circle) or mice with CHF (solid circle). Results are expressed as mean±SEM. **P<0.01 by repeated-measures ANOVA. Acetylcholine: normal, n=15; CHF, n=10. Flow: normal, n=24; CHF, n=23.

**Figure 2.** Effect of sodium orthovanadate, a nonselective PTP inhibitor, on the response to flow in arteries taken from normal (n=7; left) or CHF mice (n=10; right). Responses to increased intraluminal flow were assessed before (open circle) or after (solid circle) incubation with sodium orthovanadate ($10^{-5}$ mol/L) for 30 minutes. Results are expressed as mean±SEM. **P<0.01 by repeated-measures ANOVA.
isolated from CHF mice and treated with the PTP1B inhibitors are shown in Figure 6. In these arteries, FMD was markedly reduced by the PI3K and NOS inhibitors.

Expression of PTP1B in Mesenteric Arteries
Real-time quantitative PCR experiments demonstrated that mice mesenteric arteries expressed PTP1B mRNA; however, this expression was not affected by CHF (Figure 7, left).

Western blotting experiments also showed that PTP1B was present in mesenteric arteries to the same extent in normal and CHF mice (Figure 7, right). PTP1B could also be detected by Western blotting in cultured (coronary) endothelial cells (Figure 7, right).

Immunolocalization experiments demonstrated that PTP1B was present in the endothelium of mice mesenteric arteries. PTP1B was also present in the adventitia but was not detected in the media (Figure 7, right).

Effects of CHF and PTP1B Inhibition on the Phosphorylation Pathways of NO Production
In arteries isolated from normal mice, flow (200 μL/min) induced a transient phosphorylation of eNOS on serine 1177,
with a peak after 2.5 to 5 minutes of flow. This flow-induced eNOS phosphorylation was virtually abolished in arteries isolated from CHF mice. In those arteries, however, the PTP1B inhibitor AS279 (10^{-5} \text{mol/L}) induced a marked, transient serine phosphorylation of eNOS that peaked after 2.5 minutes of flow. In contrast, neither CHF, flow, nor AS279 affected the levels of eNOS in the same experimental conditions (Figure 8A).

Compared with normal (sham-operated animals), CHF reduced the levels of Akt and P-Akt (Figure 8B). The PTP1B inhibitor AS279 (10^{-7} \text{mol/L}) increased the levels of P-Akt, without affecting Akt.

**Discussion**

The main finding of the present study is that short-term in vitro treatment with 3 structurally different, selective inhibitors of PTP1B restored endothelial dysfunction and especially flow-dependent, NO-mediated vasodilatation in peripheral resistance arteries isolated from mice with CHF. This was accompanied by a restoration of flow-mediated serine phosphorylation of eNOS in the same resistance arteries. Furthermore, PTP1B could be detected in mesenteric arteries by PCR and Western blotting and appeared to be present in the endothelium. Together, these results demonstrate that PTP1B inhibitors, by favoring tyrosine phosphorylation, restore altered endothelial NO production in the peripheral circulation in CHF. This suggests that PTP1B inhibitors may be potent treatments for endothelial dysfunction in CHF. To the best of our knowledge, ours is the first demonstration of such an endothelial protective effect by PTP1B inhibitors.

We chose to assess endothelial dysfunction in small peripheral mesenteric arteries because peripheral vasoconstriction is a major deleterious aspect of CHF, and thus these mechanisms cannot be evaluated precisely in larger arteries that do not contribute to resistance. Our rationale is that correction of peripheral endothelial dysfunction of the peripheral circulation in CHF will favor vasodilatation, decrease afterload, and thus limit the development of cardiac dysfunction.

We used a new model of CHF-induced endothelial dysfunction in mice, in which we confirm our previous findings in rats,^{11,12,14} ie, that CHF virtually abolished FMD of the peripheral arteries but only moderately affected the vasodilatory response to acetylcholine, although both responses are to a large extent mediated by NO and in a context of maintained eNOS content (assessed by Western blot). This different impairment is possibly due at least in part to the fact that these 2 stimuli trigger the release of NO through different signal transduction pathways, ie, calcium-calmodulin–dependent for acetylcholine and calcium-independent and Tyr/Ser phosphorylation–dependent for shear stress. The selective impairment of FMD thus is likely due to alterations of the phosphorylation pathways linking shear stress and eNOS activation, and this forms the basis for our hypothesis that pharmacological modulation of these pathways, especially through increased tyrosine phosphorylation, may improve endothelial function in diseases such as CHF.

We first used a nonselective inhibitor of tyrosine phosphatases, sodium orthovanadate, which had no effect in arteries from normal mice but restored FMD in mice with CHF. This suggests that an short-term rise in the levels of tyrosine phosphorylation may reverse endothelial dysfunction in this disease. Although orthovanadate appears to potently restore endothelial function, however, this probably has little therapeutic relevance because of the marked lack of selectivity of this compound. Thus, further understanding of the mechanisms of endothelial dysfunction, together with the identification of possible new therapeutic targets, requires the use of more selective inhibitors of phosphatases and especially requires knowledge of the tyrosine phosphatase isoform(s) involved in restoration of endothelial function.
Figure 6. Top, Role of PI3K and NO in the response to flow in normal mice. Responses were assessed before (open circle) or after (solid circle) incubation with the PI3K inhibitor wortmannin (10^{-6} mol/L; n=10; left) or the NOS inhibitor L-NA (10^{-5} mol/L; n=7; right). Results are expressed as mean±SEM. Middle, Role of PI3K pathway in the response to flow in arteries from CHF mice pretreated with the PTP1B inhibitors AS279 (10^{-5} mol/L; n=11; left) and AS098 (10^{-5} mol/L; n=7; right). Responses were assessed before (open circle) or after (filled circle) incubation with the PI3K inhibitor wortmannin (10^{-6} mol/L). Results are expressed as mean±SEM. Bottom, Role of NO in the response to flow in arteries from CHF mice pretreated with the PTP inhibitors AS279 (10^{-5} mol/L; n=9; left) and AS098 (10^{-5} mol/L; n=9; right). Responses were assessed before (open circle) and after (filled circle) incubation with the NOS inhibitor L-NA (10^{-5} mol/L). Results are expressed as mean±SEM. *P<0.05, **P<0.01 by repeated-measures ANOVA.
Among the numerous PTPs known to date, recently there has been great interest in PTP1B because this isoform is known to dephosphorylate the insulin receptor and because PTP1B-deficient mice show enhanced insulin sensitivity and resistance to diet-induced obesity. Thus, PTP1B inhibitors are presently developed as possible treatments of type II diabetes and obesity. Insulin is known to activate eNOS phosphorylation and NO production through PI3K and Akt in cultured endothelial cells. On the basis of the similarity in the pathways for eNOS activation by insulin and shear stress, we reasoned that PTP1B may also regulate the response to shear stress.

At present, only limited data suggest the presence of PTP1B in the vascular wall and especially in endothelial cells. Therefore, we assessed whether PTP1B was indeed present in the small peripheral arteries used in our functional study. PCR experiments showed that small mesenteric arteries express PTP1B mRNA, and the presence of the enzyme was confirmed by Western blotting. Moreover, immunolocalization showed that the enzyme was indeed present in the endothelium of the small mesenteric arteries (together with the adventitia). This presence of PTP1B is thus compatible with the hypothesis for a role of PTP1B inhibitors in the treatment of endothelial dysfunction.

CHF did not appear to modify the expression of PTP1B, apparently ruling out the hypothesis that an overexpression of PTP1B may contribute to endothelial dysfunction. This favors the hypothesis that the impaired NO production in CHF reflects defective phosphorylation rather than increased dephosphorylation of tyrosine residues. It should be noted, however, that the quantitative changes in vascular PTP1B expression were assessed in whole arteries, and thus it is possible that subtle changes in expression, limited to the endothelium, may have remained undetected in our experiments.

We used 3 recently synthesized PTP1B inhibitors that show relative selectivity toward PTP1B (Table 1). Unlike orthovanadate, which is known to interfere with adenosine triphosphatases, protein kinases, ribonucleases, and phosphatases, these organic compounds showed no inhibitory activity on a set of randomly chosen other (non-PTP) enzymes and receptors (data not shown). Our initial experiments focused on AS279 and AS098, which were chosen because of their good potential for bioavailability, in the prospect of future in vivo studies. These 2 compounds also displayed inhibitory activity against SHP2, however, a tyrosine phosphatase that was recently shown to contribute to the activation of eNOS by shear stress. Therefore, we tested another compound, AS713, which shows a high selectivity on PTP1B over SHP2 (Table 1). At the concentration of \(10^{-6}\) mol/L (ie, 8 times below IC\(_{50}\) for SHP2), AS713 significantly restored FMD, suggesting that the observed effect is at least to a large extend dependent on PTP1B (and not SHP2) inhibition. Furthermore, recent experiments performed in carotid arteries isolated from normal mice show that inhibition of SHP2 inhibits FMD. Thus, it is unlikely that the SHP2 inhibitory effects induced by the tested compounds contribute to the increased FMD that we observed in our study.
Our results demonstrate that 3 selective, structurally unrelated PTP1B inhibitors acutely increase FMD in arteries isolated from mice with CHF, demonstrating for the first time that PTP1B inhibition may improve endothelial dysfunction. The fact that this improvement was reduced by eNOS and PI3K inhibitors suggests that the PTP1B inhibitors improved FMD by stimulating NO production through the PI3K/Akt pathway.

Our results were obtained in a model of ischemia-induced CHF, whereas endothelial dysfunction has been observed after both ischemic and nonischemic cardiomyopathy. At present, however, although the coronary consequences of nonischemic cardiomyopathy are well described, little is known about the changes occurring in peripheral arteries and about the mechanisms of endothelial dysfunction in these conditions. Thus, whether our observations extend to nonischemic heart failure is unknown and requires further investigation.

To further understand the mechanisms of the effect of the PTP1B inhibitors on endothelial function, we performed Western blot experiments on small mesenteric arteries to assess the levels of Akt and eNOS phosphorylation. At present, most of our knowledge about the phosphorylation pathways involved in eNOS activation comes from studies in cultured endothelial cells. To the best of our knowledge, our study is the first to assess eNOS phosphorylation in physiologically relevant conditions, ie, in small arteries involved in the control of vascular resistance, blood flow, and blood pressure.

We found that shear stress indeed induced a transient increase in eNOS phosphorylation on serine 1177, which was maximal after 2.5 to 5 minutes. This phosphorylation was absent in CHF, suggesting that the decreased FMD in CHF may thus be due at least in part to an impaired serine phosphorylation of eNOS. The PTP1B inhibitor increased eNOS phosphorylation at baseline (in the absence of flow) and induced a marked phosphorylation of eNOS that peaked after 2.5 minutes of flow, although this phosphorylation appeared more transient than that observed in normal conditions (as observed by the decreased phosphorylation at 5 minutes in treated CHF arteries but not in control arteries). In contrast, neither CHF nor flow nor PTP1B inhibition affected the levels of eNOS, ruling out the possibility that the changes observed in P-eNOS may be due to differences in the amount of the parent protein eNOS present in the arteries.

Interestingly, we found that CHF decreased vascular levels of Akt. It should be stressed, however, that these measurements were performed in whole arteries, and thus it is not possible to assess whether the changes in Akt occur within the endothelium. Thus, whether the decreased Akt is a limiting factor for eNOS activity and contributes to endothelial dysfunction is unknown and cannot be answered from the present study. In parallel, CHF also reduced levels of phosphorylated Akt; however, because Akt was also reduced, it is unclear whether this reflects a true alteration of Akt phosphorylation. Nevertheless, PTP1B markedly increased levels of phosphorylated Akt in this context of reduced Akt. Thus, in CHF arteries, the increased FMD may involve an increased Akt phosphorylation, leading to restored Akt-mediated phosphorylation of eNOS.

At present, the exact molecular targets of tyrosine phosphorylation involved in the endothelial response to PTP1B are still unknown. Among the proteins regulated by tyrosine phosphorylation, several have been shown to contribute to shear stress–mediated activation of eNOS, including Grb2-associated binder 1,37 platelet–endothelial cell adhesion molecule-1,30–32 vascular endothelial growth factor receptor 2,33 and heat shock protein 90.34–36 The determination of the molecular targets of tyrosine phosphorylation after PTP1B inhibition will be the subject of further investigations.

Conclusion and Perspectives
PTP1B inhibitors are presently being tested in humans as a potential treatment for type II diabetes. Our data suggest that these inhibitors may have an additional, highly therapeutically relevant property in being potent treatments for endothelial dysfunction. Given the role of the endothelium in the regulation of vascular tone, this endothelial protection may have important consequences in terms of vascular resistance and thus afterload in heart failure, and PTP1B inhibition may induce long-term beneficial effects in this disease. We are now planning to test this hypothesis in further experiments with chronic, in vivo administration of PTP1B inhibitors.

Moreover, given the role of endothelium-derived NO in the regulation of platelet and leukocyte function, such an endothelial protection may be important in terms of prevention of thrombotic events and atherosclerosis in diseases such as diabetes and also possibly hyperlipidemia or hypertension. It would therefore be important to extend our observation to other diseases that are also associated with endothelial dysfunction.

Acknowledgments
We thank Sylvanie Renet and Françoise Lallemant for their help with PCR and histology, Jean-Paul Morin for his help in setting up the perfused arteries and cultured cells, and Rosanna Pescini Gobert for her help in setting up the selectivity assays.

Sources of Funding
This work was supported in part by a grant from the Fondation de France. Magali Vercauteren is the recipient of a scholarship from the Conseil Régional de Haute Normandie, which also funded the Western blotting equipment. Dr Richard is the recipient of a research grant from Serono Pharmaceuticals.

Disclosures
Drs Hooft van Huijstuijlen and Bombrun are employees of Serono Pharmaceuticals Research Institute. The remaining authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Chronic heart failure (CHF) induces peripheral arterial vasoconstriction, which then increases afterload and aggravates heart failure. This peripheral constriction may be due in part to endothelial dysfunction and especially altered nitric oxide (NO) production. Thus, prevention of endothelial dysfunction and restored NO production may be beneficial in CHF. Using a mouse CHF model, we identified a novel way to increase NO production, using inhibitors of protein tyrosine phosphatase 1B (PTP1B). Indeed, short-term in vitro incubation with various PTP1B inhibitors restored flow-mediated, endothelium-dependent vasodilatation of small peripheral arteries of CHF mice. Western blot experiments revealed that PTP1B was indeed present in endothelial cells and that its inhibition, by increasing cellular levels of phosphorylated tyrosine, favored serine phosphorylation of endothelial NO synthase, resulting in NO-mediated responses. Our results identify for the first time PTP1B inhibitors as new treatments for endothelial dysfunction in CHF but also possibly in other diseases.
Improvement of Peripheral Endothelial Dysfunction by Protein Tyrosine Phosphatase Inhibitors in Heart Failure
Magali Vercauteren, Elise Remy, Corinne Deyaux, Brigitte Dautreaux, Jean-Paul Henry, Fabrice Bauer, Paul Mulder, Rob Hooft van Huijsguijnen, Agnès Bombrun, Christian Thuillez and Vincent Richard

_Circulation_. 2006;114:2498-2507; originally published online November 13, 2006; doi: 10.1161/CIRCULATIONAHA.106.630129

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/23/2498

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